Akademik Ziraat Dergisi 3(2):53-60 (2014)
ISSN: 2147-6403 http://azd.odu.edu.tr

Research
(Araştırma)

Multi regression analysis of the effect of potassium bicarbonate on *in vitro* the mycelial growth and sclerotial germination of *Botrytis cinerea*

Muharrem TÜRKKAN[1], İsmail ERPER[2]

[1]Ordu University, Faculty of Agriculture Department of Plant Protection, 52200 Ordu
[2]Ondokuz Mayıs University, Faculty of Agriculture Department of Plant Protection, 55270 Samsun

Received: 31 October 2014, Kabul tarihi: 17 February 2015
Corresponding author: Muharrem TURKKAN, e-mail: muharremturkkan@odu.edu.tr

Abstract

In this study, the effects of time and 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. ANOVA and multi-regression analysis showed a close relationship between actual and predicted mycelial growth and sclerotial germination of *B. cinerea*. The models developed to predict mycelial growth and sclerotial germination were, respectively, MG=(a)–(b x T2) + [c x (D x T)] and SG=(a)+(b x T)–[c x (D x T)]+[d x (D^2 x T)], where a, b, c and d represent co-efficients obtained through multi regression analysis, T represents time and D represents dosage. R^2 values of mycelial growth and sclerotial germination were 0.83 and 0.81, respectively. Standard errors were found to be significant at $p<0.001$.

Anahtar kelimeler: *Botrytis cinerea*, potassium bicarbonate, multi-regression analysis

Botrytis cinerea'nın *in vitro* miselyal gelişmesi ve sklerotial çimlenmesi üzerine potasyum bikarbonatın etkisinin çoklu regresyon analizi

Öz

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, *B. cinerea*'nın gerçek ve tahmin edilen miselyal gelişme ve sklerotial çimlenme değerleri arasında yakın bir ilişki olduğunu göstermiştir. Sırasıyla, miselyal gelişme ve sklerotial çimlenmeyi tahmin etmek için geliştirilen modelde MG=(a)–(b x T2) + [c x (D x T)] and SG=(a)+(b x T)–[c x (D x T)]+[d x (D^2 x T)] a, b, c ve d çoklu regresyon analizinden elde edilen katsayıları, T zamanı ve D dozu temsil etmektedir. Miselyal gelişme ve sklerotial çimlenmenin R^2 değerleri sırasıyla 0.83 ve 0.81'dir. Standart hatalar $p<0.001$ seviyesinde önemli bulunmuştur.

Key words: *Botrytis cinerea*, potasyum bikarbonat, çoklu regresyon analizi

Introduction

Botrytis cinerea Pers. Fr. causes pre- and post-harvest diseases, attacking flowers, leaves, stems, fruit and other parts of many plants species, including kiwifruit, grapes, apples, strawberries, tomatoes, cucumbers, peppers, bulb flowers, and ornamental plants (Agrios, 2005; Elad et al., 2007). On kiwifruit, the pathogen commonly causes post-harvest stem-end rot of harvested fruit during cold storage (Brook, 1991; Michailides and Elmer, 2000) and has been found on kiwifruit in countreis as widespread as Italy (Bisiach et al., 1984), Japan (Ieki, 1993), the United States, New Zealand (Michailides

and Elmer, 2000) and Turkey (Karakaya and Bayraktar, 2009).

Grey mold disease caused by *B. cinerea* is controlled mainly by pre- and post-harvest fungicide treatment (Bulit and Dubos, 1988). However, not only do residues of most traditional fungicides pose health risks to humans and have a negative effect on the environment, *B. cinerea* can easily develop races with high resistance against many fungicides (Erkan et al., 1997; Palmer et al., 1997; Yıldırım and Yapıcı, 2007). As a result, there is a need to develop safe and effective ways of controllng post-harvest decay of different plant species that are also economical and compatible with commercial handling (Karabulut et al., 2005). Bicarbonate compounds represent one of the best alternatives to traditional pesticides. Bicarbonates are widely utilized in the food industry to regulate pH, avoid undesirable fermentation and improve texture and taste. They also have wide-spectrum antimicrobial properties and 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; Türkkan, 2014).

Predictive models are commonly explored using computational or simulation techniques (Odabaş et al., 2009). Simulation software may be general-purpose, i.e. intended to capture a variety of developmental processes that may vary according to input files, or special-purpose, i.e. intended to capture a specific phenomenon. Input data range may from a few parameters, for example, in models describing a basic mechanism, to thousands of measurements, for example, in calibrated descriptive models of specific plants. Standard numerical outputs (i.e. numbers or plots) may be complemented by computer-generated images and animations (Çalışkan et al., 2010).

Given the difficulties generally involved in studying fungi in their natural habitats by experimental methods alone, there is an important need for models that can be used in the epidemiological analysis of fungal plant pathogens. Mathematical modeling is an efficient tool for assessing how individual or combined environmental factors affect microorganisms that degrade processed foods. Quantifying the relationships among fungi, plants and environmental factors such as temperature, pH and water activity (a_w) in terms of disease development using quantitative models can aid in the design and use of pathogen management strategies. The field of predictive microbiology has developed various models 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). In one study, Cuppers et al. (1997) modelled mold growth on a solid culture medium at various temperatures and NaCl concentrations using five common food spoilage molds. In another study, Lahlali et al. (2007) developed mathematical models to capture the effects of three incubation temperatures and six water activities (a_w) on in vitro growth rates of the pathogen *B.cinerea*; within the limits of the experiment, all models proved to be good predictors of *B. cinerea* growth rates. Judet-Correia et al. (2010) also developed and validated a model for predicting the combined effect of temperature and aw on the radial growth rate (μ) of *B. cinerea* and *Penicillium expansum* on grape berries.

To our knowledge, there is no available literature on the mathematical modelling of mycelial growth and sclerotial germination of *B. cinerea* exposed to potassium bicarbonate ($KHCO_3$). Therefore, this study aimed to develop a model for estimating the mycelial growth and sclerotial germination of *B. cinerea* in vitro exposed to increasing doses of $KHCO_3$ at different times using multiple linear regression analysis.

Materials and Methods

Fungal culture

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

Assesment of mycelial growth

Potassium bicarbonate concentrations of 0, 10, 20, 30, 40, 50, 60, 70, 80 and 90 mM ($KHCO_3$; Carlo Erba Reagenti (Milan, Italy) with pH values of 6.0, 7.4, 7.8, 7.9, 8.0, 8.1, 8.1, 8.1, 8.2 and 8.2, respectively, were added to autoclaved PDA medium cooled to 50°C and dispensed aseptically into 9-cm-diameter petri plates. A mycelial disc (5 mm dia.) taken from 7-day-

old *B. cinerea* culture grown on PDA was placed in the center of each $KHCO_3$-amended PDA plate. Plates were sealed with parafilm and incubated at 25°C for 6 days. Growth of each fungal colony was measured daily (at 0, 24, 48, 72, 96, 120, 144 hrs) (Ordonez et al., 2009). All experiments were repeated twice.

Assesment of sclerotial germination

Potassium bicarbonate concentrations of 0, 10, 20, 30, 40, 50, 60, 70, 80 and 90 mM with pH values of 6.0, 7.4, 7.8, 7.9, 8.0, 8.1, 8.1, 8.1, 8.2 and 8.2, respectively, were added to autoclaved PDA medium cooled to 50°C and dispensed aseptically into 9-cm-diameter petri plates. Sclerotia obtained from pure cultures of *B. cinerea* grown on PDA were surface-disinfected to prevent proliferation of bacterial contaminants by placing them in 70% ethanol for 40 s, rinsing in sterile-distilled water (SDW), adding a solution of streptomycine (200 µg/ml), and storing them at 4°C overnight. Sclerotia were blotted dry on sterile paper towels to eliminate excess antibiotic solution, and 10 sclerotia were placed on each of the petri plates containing $KHCO_3$-amended PDA (Ordonez et al., 2009). Plates were incubated at 25°C for 6 days, and the number of germinated sclerotia was recorded daily (at 0, 24, 48, 72, 96, 120, 144 hrs) for each treatment. All experiments were conducted twice.

Experimental design and data analyses

All experiments were conducted in a completely randomized design with 10 treatments and 3 replications. Analysis of variance (ANOVA) was performed using the program Minitab (version 12, "Minitab", USA), and Duncan's test was used to compare treatment means, with level of significance set at 0.05.

Model Construction

Data obtained for *B. cinerea* mycelial growth and sclerotial germination was subjected to multiple regression analysis. The R software program was used to identify the equations that produced the best estimates of *B. cinerea* mycelial growth and sclerotial germination. Models were constructed using various subsets of the independent variables "dose (mM)" and "time". The best estimates were obtained for mycelial growth (MG) using the formula MG = (a) – (b x T2) + [c x (D x T)] and for sclerotal germination (SG) using the formula SG = (a)+(b x T)–[c x (D x T)]+[d x (D^2 x T)], where, a, b and c are co-efficients obtained through multiple regression analysis (Table 1 and Table 2), T is time of sclerotial germination and D is dose. Multiple regression analysis was carried out until the least sum of square (R^2) was obtained. 3-D graphics were constructed using the software program Slidewrite.

Results and Discussion

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

Mycelial Growth of *Botrytis cinerea*

Mycelial growth of *B. cinerea* began within 24 h of incubation at 25°C. However, growth was significantly curtailed with increasing concentrations of $KHCO_3$. *B. cinerea* completely covered the petri dishes at 0 mM $KHCO_3$ concentrations at 96 h, compared to 120 h at 10-20 mM $KHCO_3$ concentrations. Mycelial growth was strongly inhibited at concentrations greater than 50 mM and completely inhibited at concentrations of 90 mM (Fig 1). These findings are supported by previous studies reporting similar results with regard to 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.

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 T2) + [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.

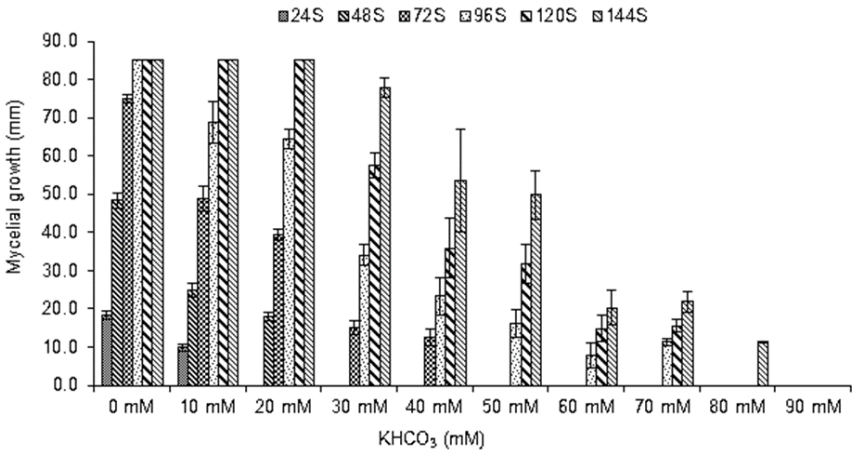

Figure 1. Mycelial growth of *Botrytis cinerea* on petri dishes exposed to increased concentrations of potassium bicarbonate ($KHCO_3$) at different times.

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)–(2.581xT2)-[0.203x(AxT)]

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. The R^2 value of 0.83 obtained in this study for mycelia growth indicates that the model developed captures 83% of actual reality.

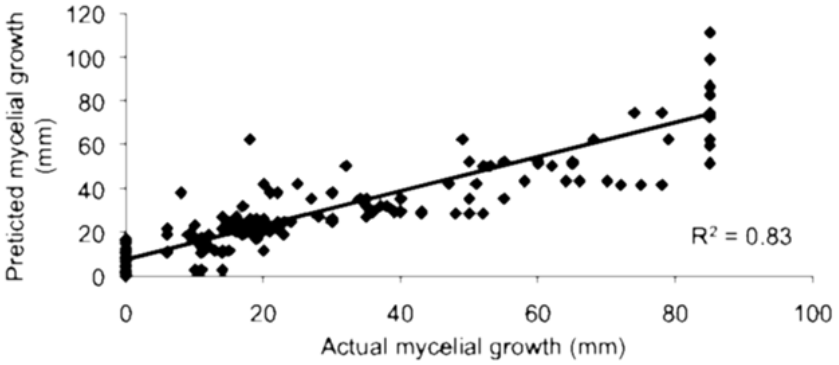

Figure 2. Relationship between actual and predicted mycelial growth of *Botrytis cinerea.*

The effects of dose and time on *B. cinerea* mycelial growth are shown in Figure 3, which was created using the mathematical equation as input in the Slidewrite graphics program, which can produce 3-dimensional graphics using values entered directly as well as those obtained through a mathematical equation. As Figure 3 clearly shows, the rate of mycelial growth of *B. cinerea* changes in line with increases and decreases of potassium bicarbonate.

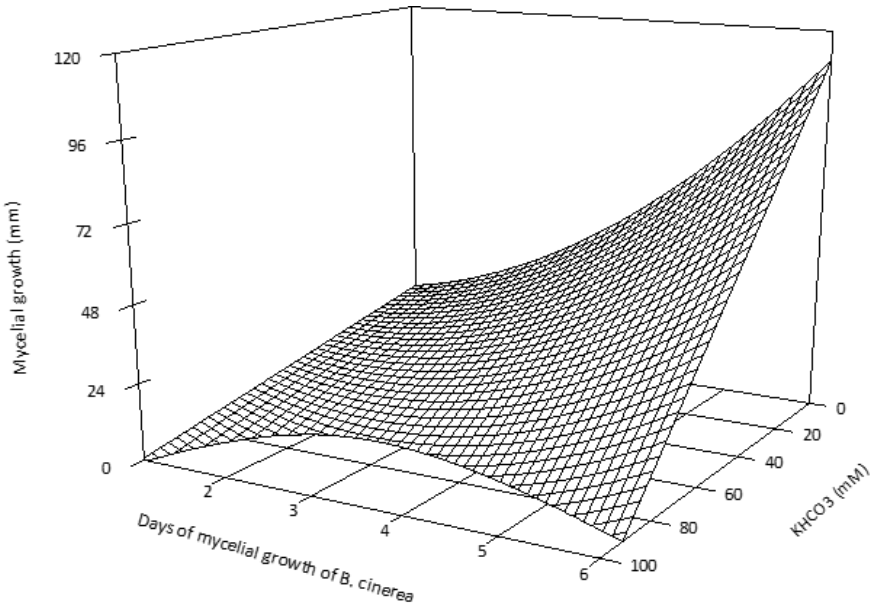

Figure 3. Mycelial growth of *Botrytis cinerea* on petri dishes exposed to increased concentrations of potassium bicarbonate ($KHCO_3$) at different times.

Sclerotial germination of *Botrytis cinerea*

Sclerotial germination of *B. cinerea* was significantly affected by potassium bicarbonate concentration. Although only the control group (0mM) showed sclerotial germination at 48 h, at the end of six days, sclerotial germination was not observed at concentrations of 40 mM and above (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).

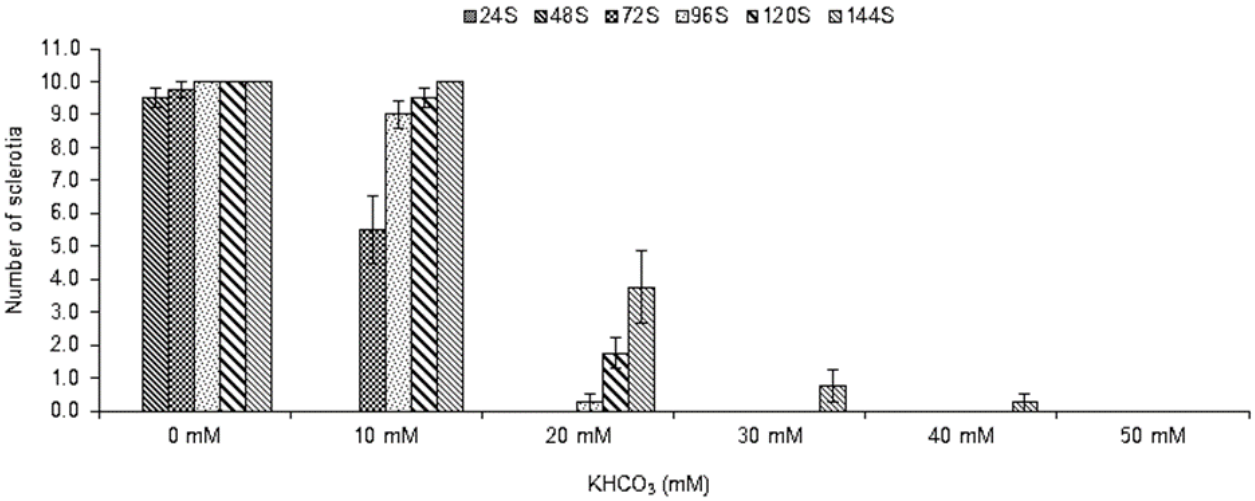

Figure 4. Number of sclerotia of *Botrytis cinerea* exposed to different concentrations of potassium bicarbonate ($KHCO_3$) after 144 h in vitro.

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)+(2.395704xT)–[0.11392x(DxT)]+[0.00134x(D^2xT)]

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.

Previous studies have also used mathematical modelling in the epidemiological analysis of pre-and-post-harvest plant pathogens as an efficient method for evaluating how individual or combined environmental factors affect microorganisms that degrade processed foods.

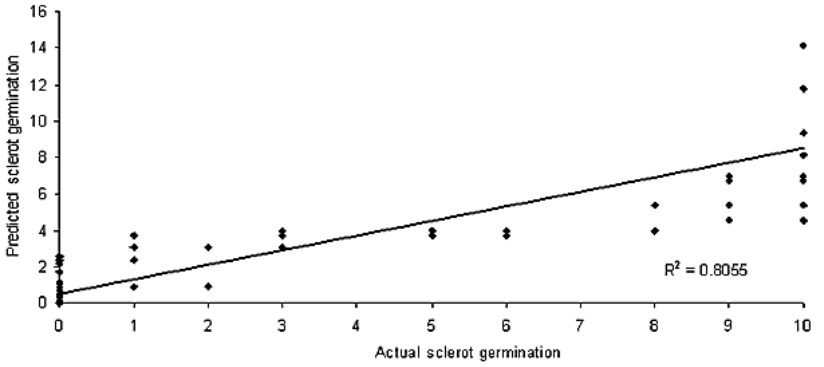

Figure 5. Relationship between actual and predicted sclerotial germination of *Botrytis cinerea*.

Predictive microbiology has produced various models for establishing 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). These include models of five common food spoilage molds (*Penicillium roqueforti*, *Trichoderma harzianum*, *Paecilomyces variotii*, *Aspergillus niger* and *Emericella nidulans*) on a solid culture medium at various temperatures and NaCl concentrations (Cuppers et al. 1997). Lahlali et al. (2007) also developed and validated models for predicting the in vitro effect of water activity (a_w) and temperature on the radial growth of *B. cinerea* at three incubation temperatures and six water activities. Ultimately, within the limits of their study, all models proved to be good predictors of *B. cinerea* growth rates (g, mm d−1) and confirmed the general finding that fungal growth is influenced more by aw than by temperature. In another study in which a model was developed for predicting radial growth rate (μ) of *B. cinerea* and *Penicillium expansum* on grape berries using the factors temperature and aw, Judet-Correia et al. (2010) demonstrated the usefulness of the gamma concept for validating predictive models relating to agricultural products and foodstuffs.

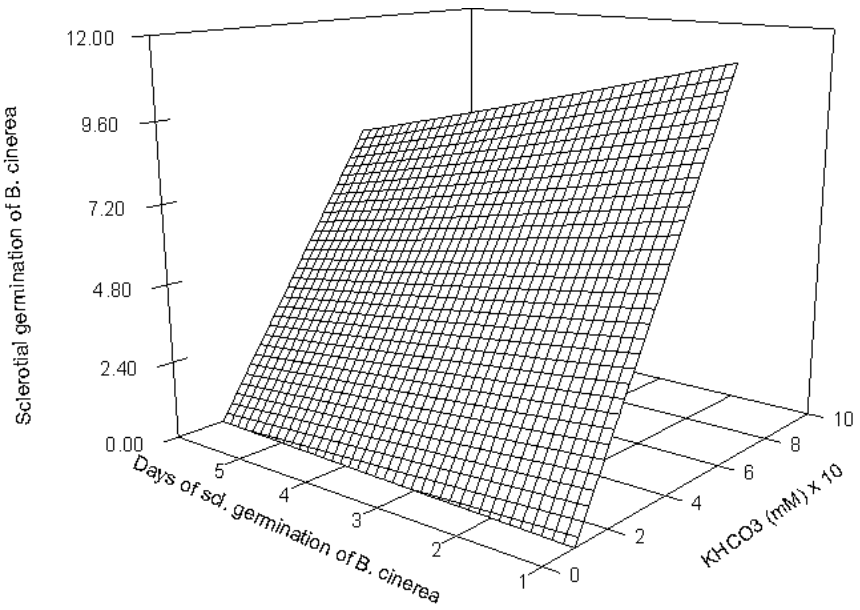

Figure 6. Sclerotial germination of *Botrytis cinerea* on petri dishes exposed to increased concentrations of potassium bicarbonate ($KHCO_3$) at different times.

That study used grape analogues to validate the combined effects of temperature and water activity on growth and then, once the model was validated, determined the optimum growth rate of grape berries. This approach allowed for validation of the model over a wide range of temperatures and water activities as well as estimation of the optimal growth rate of grape berries under non-optimal conditions. Contrary to the main values, optimum growth rates weres shown to be strongly dependent upon the strain and the medium.

Prior to the present study, there has been no published study reporting on the use of mathematical modeling (multi-regression analysis) to evaluate the effects of time and $KHCO_3$ dosage on mycelial growth and sclerotial germination of *B. cinerea*. Results of ANOVA and multiple regression analysis demonstrated a close relationship between actual and predicted mycelial growth and sclerotial germination of *B. cinerea* using the models developed in the present study (Fig 2, Fig 5).

Conclusion

This study used multiple linear regression analysis to evaluate the relationship between *B. cinerea* mycelial growth and sclerotial germination (dependent variables) and $KHCO_3$ dose and time (independent variables). Regression co-efficients for *B. cinerea* mycelial growth and sclerotial germination were found to be 0.83 and 0.81, respectively. The high predictive value of the models constructed for predicting mycelial growth and sclerotial germination suggest that mathematical modeling can contribute to the epidemiological analysis of other important fungal pathogens. Additionally, the use of mathematical modeling to quantify the roles of environmental factors in the development of fungal disease can be of help in the design and implementation of management strategies for other fungal post-harvest plant pathogens.

Acknowledgment

The authors gratefully acknowledge the contributions of Dr. Mehmet Serhat Odabaş in assisting with mathematical modeling.

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