

Aresponse Surface Methodology Approach to Determine the Influence of Some Environmental Factors on Mycelial Growth and Spore Production of *Didymella pinodes*

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Abstract: Ascochyta blight caused by *Didymella pinodes* (Berk. et Blox.) Vesterg. can cause severe damage in pea. The objectives of the present study were to determine the optimum conditions for both the growth and spore production of *D. pinodes in vitro* on PDA medium using the response surface methodology. Overall, both the growth and sporulation were significantly influenced by the three environmental factors tested. Mycelial growth was optimal at 20° C and decreased at 30° C. Similarly, the spore production was maximum at 20° C and ceased at 30° C. The fungus grew at three pH levels with no sporulation at 8.1. The pH of 6.8 was the best for both growth and sporulation the relative humidity (RH) of 85% and 95% was favorable for both sporulation and growth with optimal response at 95%. There was positive interaction between the pH and temperature and between temperature and RH. The quadratic regression model was checked using the coefficient of determination R², the adjusted R²_{adj} and the multiple correlation coefficient R. All the three parameters were revealed high (R² = 0.933; R=0.966) which indicates high significance of the model and the correlation between the experimental data and the predicted values.

Key words: Response surface methodology • pH • Temperature • *Didemylla pinodes* • Relative humidity • Growth • Sporulation

INTRODUCTION

Didymella pinodes (Berk. & Blox.) Vesterg. is one of the most destructive pathogens of pea that causes Ascochyta blight on pea [1]. It is widespread throughout the major pea growing areas worldwide [2-4]. The disease causes yield losses of over 50 per cent in Canada during favourable conditions [5-7]. In view of its complexity and economic importance, the disease has been investigated in many pea-growing regions around the world. In recent years, an increased incidence of Ascochyta blight has been seen in several production areas in Algeria and has led to increasing yield loss [7, 8]. It is therefore important to understand effects of environmental factors on the epidemiology of *D. pinodes*. Among the various factors, temperature and relative humidity have significant effect on the life cycle and other components of disease cycle [9-11]. The traditional approach to measure the effect of

biological effect based on one factor at a time, commonly abbreviated OFAT, is less accurate method compared to the response surface methodology [12]. It is less efficient than a factorial screening design and can provide incorrect conclusions in case of strong interactions among the factors. Hence, in this study, optimum conditions for mycelial growth and sporulation were determined using the response surface methodology (RSM). RSM is a compilation of statistical techniques widely used in engineering field and now extensively used in microbiology to determine the effects of parameters on the optimum conditions of biological phenomenon [13-15]. This technique gives contour plots from linear, interaction and quadratic effects of two or more parameters and fits the experimental data to calculate the optimal response. This technique has been widely used to investigate the optimization of physiochemical parameters and factors of several biotechnological processes [13, 16]. In this study,

RSM was adopted to measure the optimal conditions of *D. pinodes* (mycelial growth and spore production) in the presence of various environmental factors and their interactions.

MATERIALS AND METHODS

Fungal Material and Media Preparation: An isolate of *D. pinodes*, tn0203 used in this study was originally isolated from infected pea plants (cv. Onward). The strain was raised on potato dextrose agar (PDA) medium at 22° C. Medium was poured into glass Petri dishes inoculated with 5-mm-diameter plugs of mycelium cut from the edge of an actively growing colony under aseptic condition.

Linear Growth Measurements and Sporulation Assessments: Final measurement of linear growth and sporulation was recorded after 10 days of incubation. Conidia from 10-day old culture were collected by adding 10ml of sterile distilled water to dislodge spores. One drop of this spore suspension was placed on a haemocytometer and the number of spores were counted.

Effect of Temperature and Relative Humidity on Mycelial Growth and Sporulation of *D. Pinodes* Isolates: Mycelial growth was evaluated on PDA. Inoculated plates were incubated at 10, 20, 25 and 30°C, in the dark. There were three replicates of each treatment. Observations on colony growth in diameter were recorded after 10 day of incubation. For the influence of the relative humidity studies, the inoculated plates were exposed to 50, 80 and 95 per cent relative humidity levels (Bretag et al, 2006; Tivoli and Banniza, 2007). The desired humidity were obtained by using respective salt solutions and acids.

Effect of Ph on Mycelial Growth and Sporulation of *D. Pinodes* Isolates: Mycelial growth were studied at 3 pH levels 4.1, 6.8 and 8.1. The desired pH was obtained by adding the required amount of buffer 0.1 N Citric acid or 0.1 N NaOH with the help of a digital pH meter. Then medium was sterilized in autoclave at 120°C for 15 min. A five mm mycelial disc was transferred from the margin of active growing colony, to the centre of each PDA plate. Final linear growth and sporulation were undertaken according to already described method.

Statistical Analysis: A completely randomized factorial design with three replicates (4×3×3×3) was used. Analysis of variance (ANOVA) was first performed to determine the

effect of each factor on both growth and sporulation of *D. pinodes*. On the other hand, a regression design was as a mathematic function. Since the conventional approach known as « one factor at one time » is often employed for such data is in our view more laborious and statistically less informative. The reason why, the collected data were subjected to the surface response analysis to fit a second order regression model of growth and sporulation under different levels of temperatures, relative humidity and pH conditions. The responses can be simply related by linear, or quadratic models, which also includes the linear model is given as follows:

$$\eta = \beta_0 + \sum_{j=1}^k \beta_j x_j + \sum_{j=1}^k \beta_{jj} x_j^2 + \sum_{i < j=2}^k \sum_{i} \beta_{ij} x_i x_j + e_i \quad (1)$$

where, η is the response; x_i and x_j are variables; β_0 is the constant coefficient; β_j 's, β_{ij} 's and β_{jj} 's are interaction coefficients of linear, quadratic and the second-order terms, respectively; e_i is the error. In this study, the quality of the fit of polynomial model was expressed by the coefficient of determination R^2 and R^2_{adj} . The statistical significance was checked with adequate precision ratio and by the F-test. The results of the experimental design were analyzed and interpreted Statistica version 6 software. The variables of the experiments were coded according to the following equation (2) :

$$x_i = \frac{X_i - X_0}{\Delta X}$$

where x_i , stands for the coded value of the i^{th} variable; X_i , stands for the real value of an independent variable; X_0 , stands the real value of an independent variable at the center point.

The results of the experimental design and interpreted using STATISTICA (version 6.0).

RESULTS

The quantitative assessments of mycelial growth and spore production of *D. pinodes* under different combinations conditions of temperature, pH and RH were investigated.

The summary of the two-way analysis of variances ANOVA revealed significant effect of abiotic factors on the radial growth ($P < 0.0001$) and sporulation ($P < 0.0001$) of the tested fungus. Furthermore, a significant interaction was noted between temperature and pH for

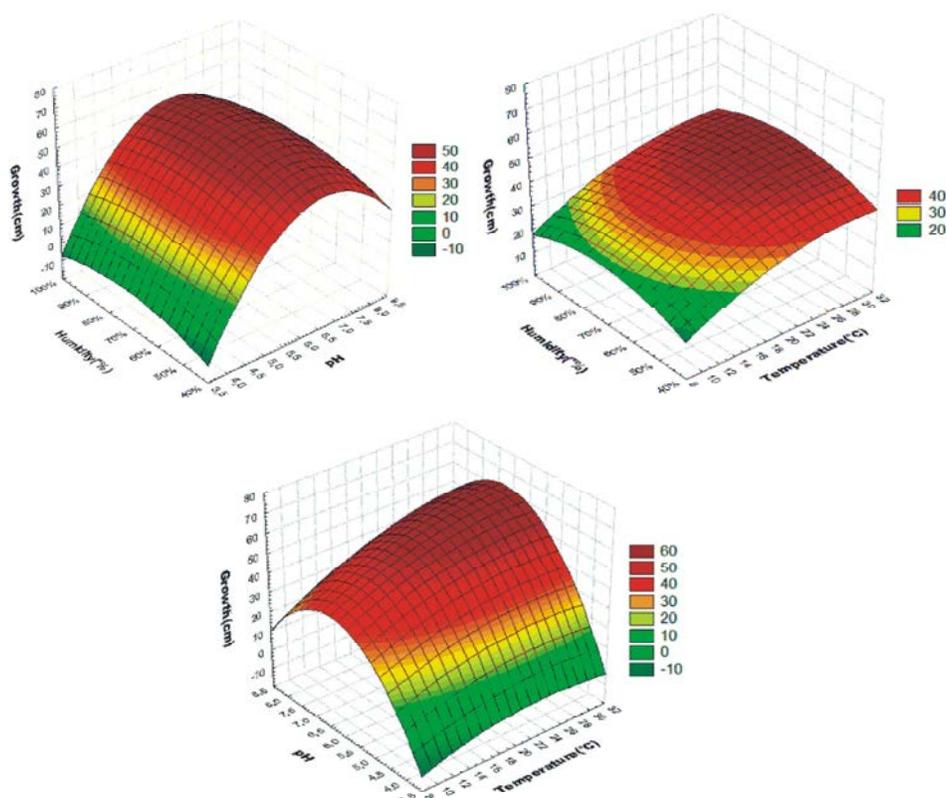


Fig. 1: Three dimensional surface plot for:

- a) the effect of temperature and pH on the mycelial growth of *D. pinodes*.
- b) the effect of temperature and pH on the mycelial growth of *D. pinodes*.
- c) the effect of temperature and pH on the mycelial growth of *D. pinodes*

both the growth and spores production. However, no significant effects was observed between pH and RH%. Linear growth on natural PDA showed a variable trend in response to change in temperature. Mycelial growth increased as temperature increased from 10°C to 20°C and then decreased with further increase in temperature (Fig. 1a-c). At 30°C, the growth declined markedly.

Similarly, spore production showed same trends as radial growth with respect to temperature changes. The optimum temperature for sporulation on natural PDA was recorded at 20° C. The sporulation decreased with further increase in temperature. At 30° C, the *D. pinodes* isolate failed to sporulate. Linear growth of *D. pinodes* isolate was studied at three pH levels. The results revealed significant differences between the three pH levels. The optimum growth was observed at 6.8 followed by the pH 4.1 and then pH 8.1 (Fig. 1-3). Similarly, spore production showed same trends as radial growth with respect to pH changes. The maximum sporulation was recorded at pH of 6.8. Furthermore, significant differences

were seen between the three levels of pH (P <0.0001). With regards to the effect of the relative humidity, the linear growth was significantly greater at RH of 95% as compared to the growth noted at 50% and 80% respectively. An abundant sporulation was observed at RH of 80% and 95% with an optimum at 95% (Fig. 2a-c).

The experimental data were subjected to a multiple regression analysis namely the response surface methodology was presented in the Table 3. The results of the second order polynomial regression equation were obtained using the software STATISTICA version 6. The mathematical expression of the linear growth of mycelium and the sporulation of *D. pinodes* with regards to X_1 , X_2 , X_3 are shown bellow:

$$Y_1 = -9.652 + 5.753X_1 + 1.952X_2 + 0.776X_3 - 5.858X_1^2 - 1.634X_2^2 - 0.801X_3^2 - 0.441X_1X_2 - 0.062X_1X_3 + 0.0446X_2X_3 \quad (3)$$

$$Y_2 = -8.318 + 3.967X_1 + 5.638X_2 - 0.794X_3 - 4.054X_1^2 - 5.271X_2^2 - 0.858X_3^2 - 0.128X_1X_2 + 0.030X_1X_3 + 0.115X_2X_3 \quad (4)$$

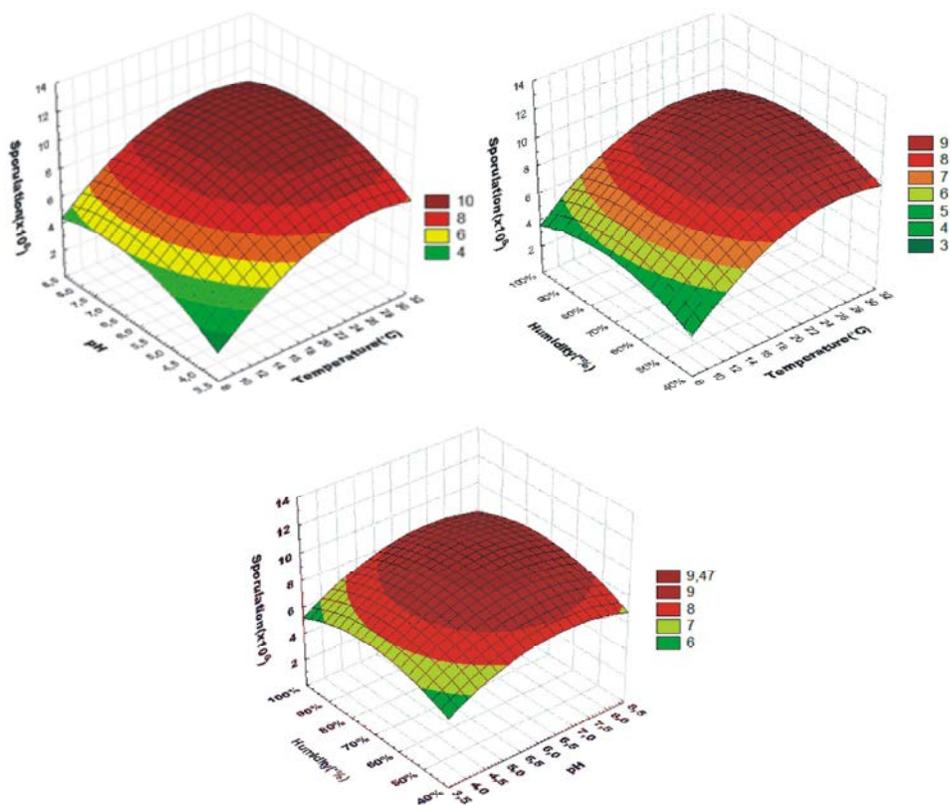


Fig. 2: Three dimensional surface plot for:
 a) the effect of temperature and pH on the sporulation of *D. pinodes*.
 b) the effect of temperature and pH on the sporulation of *D. pinodes*.
 c) the effect of temperature and pH on the sporulation of *D. pinodes*.

Table 3: Regression coefficients and their corresponding p-values for predicting optimized responses on mycelial growth and sporulation using quadratic linear regression model.

Sporulation (Y1)			Mycelial growth (Y2)		
Factor	b-coefficient	p-value	Factor	b-coefficient	p-value
Intercept	-9.652	0.00001	Intercept	-8.318	0.00001
A-temperature	5.753	0.00001	A-temperature	3.967	0.00001
B-pH	1.952	0.00001	B-pH	5.638	0.00001
C-RH%	0.776	0.00001	C-RH%	-0.794	0.0214
A ²	-5.858	0.00001	A ²	-4.054	0.00001
B ²	-1.634	0.00001	B ²	-5.271	0.00001
C ²	-0.801	0.0177	C ²	-0.858	0.163
A*B	-0.441	0.0005	A*B	-0.128	0.563
A*C	-0.062	0.6330	A*C	0.030	0.898
B*C	0.0446	0.757	B*C	0.115	0.655
Others statistics			Others statistics		
R	0.966		R	0.887	
R ²	0.933		R ²	0.788	
Adj R ²	0.927		Adj R ²	0.768	
F-value of model	152.507		F-value of model	40.592	
P=	0.0001		P=	0.00001	

Adj R²: Adjusted R². *Coefficients with p-value greater than 0.05 quadratic model equations obtained by response surface methodology.

In which Y_1 and Y_2 represent response variable of sporulation and mycelial growth of *D. pinodes* isolate respectively. X_1 , X_2 and X_3 are the values of the independent variables which indicates temperatures, pH and RH% respectively. The coefficients X_1X_3 and X_2X_3 were statistically insignificant and consequently they were deleted from the spore production response (Table 3). Similarly, the coefficients X_1X_2 , X_1X_3 and X_2X_3 were statistically insignificant for the growth response (Table 3) and hence deleted from the equation 4. Therefore, the new regression model were obtained for both the mycelial growth and sporulation and are represented in equation 5 and 6.

$$Y_1 = -9.652 + 5.753X_1 + 1.952X_2 + 0.776X_3 - 5.858X_1^2 - 1.634X_2^2 - 0.801X_3^2 - 0.441X_1X_2 \quad (5)$$

$$Y_2 = -8.318 + 3.967X_1 + 5.638X_2 - 0.794X_3 - 4.054X_1^2 - 5.271X_2^2 - 0.858X_3^2 \quad (6)$$

Quadratic effects of X_1 , X_2 and X_3 had increasing effects on the growth and sporulation response. Furthermore, the interactions of these factors were also shown using the three dimensional surface plots of the mycelial growth (Fig. 1-3) and spore production (Fig. 4-6). The regression equations 5 and 6 were then checked using the goodness of fitness by the determination coefficient (R^2) and the multiple correlation coefficient (R). In this study, the multiple regression coefficient (R) was estimated from the second degree polynomial equation. The value of R was 0.966 and 0.887 respectively for the sporulation and mycelial growth model. Both the values are closer to 1 which indicates the high correlation between the predicted and the experimental values. Moreover, the determination coefficient value R^2 was 0.933 for the sporulation model. This implies that only 6.7% of the total variation are not explained by the model.

Finally, the value of the adjusted determination coefficient (R_{adj}) was also high. It was 0.927 and 0.768 for the sporulation and mycelial growth models respectively indicating a high significance of the obtained models.

DISCUSSION

The objectives of this study were to determine the influence of the principal environment factor on *D. pinodes*, the causal agent of Ascochyta blight. The results obtained under *in vitro* conditions showed how temperature, pH and RH% affects the growth and sporulation. This study presents evidence that both

parameters are sensitive to temperature. The optimum temperature range was usually 10-20° C. At 30°C, linear growth decreased drastically and the spore production ceased. Moreover, high correlation was observed between the mycelial growth and sporulation of *D. pinodes*. In fact, growth at high temperature was unexpected because the *D. pinodes*, the causal agent of Ascochyta blight on pea is commonly found during cool season. Cochrane [17], Shew *et al* [18] and Malik and Singh [19] had already suggested that the temperature plays an important role in influencing the growth and sporulation for fungus. Cochrane [20], considered that the temperature range for sporulation is narrower than that of the vegetative growth. Similarly, Leach [21]; Meier *et al.* [22] have reported the existence of variation requirements of range of temperature between species and also within species. Previous studies had also suggested that temperature below 6C impedes infection process, whereas, temperature between 18 and 21°C favors the infection initiation [23-24].

Among the abiotic factors, pH is probably the most important environmental parameters affecting the morphological and physiological activity of numerous plant pathogenic fungi including *D. pinodes*. Our results showed that The optimum growth was observed at 6.8 followed by the pH 4.1 and then pH 8.1. These observations, suggest that the pH value of the habitat could be determinant factor for the growth and development of the fungus. Our findings, also showed a great correlation between the mycelial growth and sporulation of *D. pinodes* isolate.

Generally, most fungi grew on best around neutrality and on slightly acidic side of the neutral ([25-26]. On the other hand, Singh *et al*[27] suggest that slightly acidic pH clearly influence the mycelial growth and is considered as major regulatory factor for biomass production for fungus species. On the other hand, Limón *et al* [28] suggested that acidic pH favored fungal sporulation and linear growth than alkaline conditions.

Concerning the effects of relative humidity, the present results show that RH% is an important parameter that regulate both the growth and spore production. These findings suggest that the RH% value could be determinant for the developmental pattern of this fungus. During *in vivo* tests, relative humidity appears to act as limiting factor for the initiation of infection and also in the appearance of disease development [9]. Royle and Butler [29], suggested that relative humidity as an important factor enabling the fungal pathogen to infect aerial parts of plants. The same authors considered that weather

moisture is frequently used as an important indicator for the likelihood of an epidemics of aerial plant diseases. Moreover, Traperos-Casas and Casier [30], suggested that relative humidity is of great importance on both the latent and incubation period. In the other hand, Pederson and Morall[31], reported the existence of variation in the optimum requirements depending on the fungus species.

The physical factors interactions were estimated using the response surface methodology. This analysis showed that the quadratic polynomial model provides a good approach to predict the combined effects of the parameters tested. On the other hand, a significant negative quadratic effect of temperature and pH can be observed in the model. A positive interactions between temperature and pH and between temperature and RH% are significant. A similar approach has been used to the study the optimum conditions of culture of the food spoilage fungi such as *Penicillium spp* and *Rhizopus* [32-33]. In this study, the multiple regression coefficient (R) was 0.966 and 0.887, respectively for the sporulation and mycelial growth model. Moreover, the determination coefficient value R² was 0.933 for the sporulation model which indicates that 93.3% of the total variation can be explained using this model. Both the sporulation and growth rate predicted by the models clearly reflect the strong dependance of *D. pinodes* isolate on the temperature, pH and RH%. Hence, modelling the growth of *D. pinodes* on solid medium is considered as the first step towards predicting the behaviour of such plant fungus pathogen under field conditions. This could be also of great importance for the creation of the optimal conditions reproduction of symptoms on inoculated plants.

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