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Full Length Research Paper

Applications of response surface methodology approach to determine the effect of temperature, time of incubation and light conditions on germination and germ tube growth of *Puccinia coronata* f.sp. *avenae* urediosopores

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Crown rust caused by *Puccinia coronata* f.sp. *avenae* is the most damaging disease on oat. This work analyzed the effects of temperature and illumination regime during different time of incubation on both spore germination and germ tube growth, using both analysis of variance (ANOVA) and response surface methodology (RSM). This study reveals that the maximum of germination approached 95% under dark conditions at 20°C. Similarly, the maximum germ tube length was 125±23 µm under dark conditions at the same temperature after 18 h. Both spore germination and germ tube growth were observed over a wider temperature range of 5 to 30°C. The darkness conditions seem to enhance significantly ($P < 0.05$) both the germination and germ tube growth. After 4 h of incubation, germination was significantly higher under darkness regime at 15, 20 and 25°C than under light conditions. The effect of darkness conditions on germ tube growth paralleled its effect on germination. Furthermore, the response surface methodology (RSM) was applied to determine the optimal conditions of temperature, time and illuminations conditions for both the germination process and germ tube growth of urediospores for *P. coronata* f.sp. *coronata*. Values for the optimal germination and germ tube growth were 20°C and 8 h under darkness conditions. Moreover, urediospores of *P. coronata* f.sp. *coronata* germination and germ tube growth had followed a quadratic response function on temperature ($R^2 = 0.94$ and 0.97). On the other hand, the experimental values were in good agreement with the predicted ones and the model was highly significant with the correlation coefficient R being 0.97 and 0.98, respectively for germination and germ tube growth.

Key words: *Puccinia coronata* f.sp. *avenae,* temperature, time, illuminations conditions, germination process, germ tube growth, urediospores.

INTRODUCTION

Oat crown rust, caused by *Puccinia cornata Cda.* f. sp. *avenae* Eriks has become an important limiting factors for Oat (*Avena sativa*) for both grain and forage production in Algeria. On the other hand, this pathogen limits yields potential and grain quality in sensitive cultivars worldwide (Monson et a.l, 1986; Doehlert et al., 2001; Harder et Haber; 1992; Holland and Munkvold, 2001; Johnston et al., 2000).

Knowledge of the physiologic requirements of the pathogen is a key step to understand epidemiology of a plant disease. Although, it is known that temperature affects spore germination and infection, which in turn

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influence disease development, very little information is available on this aspect of the epidemiology (Young et al., 1978). Many authors suggest that among the environmental factors favorable for germination and penetration of the fungal spores, temperature and free moisture play a key role (Misra and Prasada, 1971; Sood and Wiese, 1974). The optimum temperature for urediospore germinate depends on the species, races and isolate studied. Uredospores of *Puccinia graminis* germinate within optimal temperature range of 15 to 22°C (Kramer and Eversmeyer, 1992) whereas, the optimal temperature for *Puccinia substiata* is 19 to 22°C. For the *Puccinia striformis*, the optimal temperature is between 15 and 25°C (Tollenaar and Houston, 1966). Therefore, those of *P. coronata* on Oat species germinate between 10 and 30°C (Kockman and Brown, 1975).

Although, the effect of temperature on germination of urediospore of *P. coronata* was studied previously (Kockman and Brown, 1975), information on the interaction of time, light and temperature on germination is not available.

Many authors suggested that the interaction between temperature and light influence the spore germination and germ tube growth and subsequently, infect the host plant by numerous rust pathogens (Givan and Bromfield, 1964; Kramer and Eversmeyer, 1992; Sood and Wiese, 1974; Subrahmanyam et al., 1988).

Traditional approach to measure the effect of biological effect based on one factor at a time, commonly abbreviated OFAT, is not as scientific as response surface methodology (RSM) (kenneth et al., 1995; Chang et al., 2006) . 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 germination and germ tube growth were determined using RSM.

RSM is a compilation of statistical and mathematical techniques widely used to determine the effects of parameters and then measure the optimum conditions for any biological phenomenon (Margarida et al., 2009). This techniques gives contours 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 (Chang et al., 2002; Chang and Lee, 2007).

In this study, RSM was adopted to measure the optimal conditions of germination and germ tube growth of urediospores, in which the effects and interactions of temperature, illumination conditions and time was evaluated.

MATERIALS AND METHODS

The uredioniospores were collected from 10 days old pustules and suspended in deionized water containing 0.05% of Tween 20 and filtered throw sterile cheese cloth. Spores were then counted with hemocytometer and the spores' concentration was adjusted to 1.5×10^5 spores/ml. A 0.1 ml of spore suspension was poured on 2% water agar plate (9 cm of diameter) containing 100 µg of chloramphenicol.

Effect of temperature and light conditions on urediospores germination and germ tube growth

Temperature used within this study included 5, 10, 15, 20, 25, 30 and 35°C were attained using temperature regulated incubator. Temperature fluctuated ±1°C from the stated value. The darkness conditions were achieved by wrapping each Petri dish with aluminum foil. Continuous illumination was provided by two cool 40 W white fluorescent lamp suspended 50 cm directly above the Petri dishes. Three replicates Petri dishes were used under each temperature light combination.

The numbers of germinated spores from three plates were counted after 4, 8, 12 and 18 h. Germination percentage of each combination after different period of incubation were determined by means of microscopic examination of 100 urediospores at 100× magnification in each of the three replicate dishes. Individual urediospores was considered germinated, if the length of the germ tube was equal to or greater than one half the spore diameters (Bosch et al., 1995).

Concerning the germ tube growth, an average of length of germ tubes at each period of incubation for different treatment were measured by means of an ocular micrometer of 20 germ tubes.

Data analysis

The experimental design used was a completely randomized design with three replicates. An analysis of variance (ANOVA) was used to determine the effect of each factor on both the germination and germ tube growth of urediospore. A response surface was also used to fit a second order regression model of germination rate and germ tube growth under both the illumination regimes and temperatures conditions. In the determination of optimal conditions for germination and germ tube growth of urediospores, the responses can be simply related to chosen factors by linear or quadratic models. A quadratic model, which also includes the linear model is given as:

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

Where, η is the response; x_i and x_j are variables; βo is the constant coefficient; $\beta_{j's}$, $\beta_{ij's}$ and $\beta_{jj's}$ are interaction coefficients of linear, quadratic and the second-order terms, respectively; e_i is the error.

In this study, percent degradation data were processed by Equation 1 including ANOVA to obtain the interaction between the process variables and the response. 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 using Design expert software (Stat Ease, 8.0.2 trial version).

RESULTS

The first step in this study was the identification of the

Table 1. Analysis of variance of the main effects and interactions of illumination, temperature and incubation time on the germination of urediospores of *P. coronata*.

Effect	Df	SS	MS	F value	Pr(>F)	Pr(>F)
Temperature	6	9160.8	1526.8	31.1924	1.393e-15	***
Time of incubation	3	4619.5	1539.8	31.4588	9.750e-12	***
illumination conditions	1	4.0	4.0	0.0822	0.02	*
Temperature* time of incubation	18	5878.6	326.6	6.6722	3.574e-08	***
Time*light	3	554.7	184.9	3.7774	0.01583	*
Residuals	52	2545.3	48.9			

SS: Sum square; MS: mean square.

Table 2. Analysis of variance of the main effects and interactions of illumination, temperature and incubation time on the germ tube length of urediospores of *P. coronata*.

Effect	Df	SS	MS	F value	Pr(>F)	Pr(>F)
Temperature	6	11903.8	1984.0	87.6271	< 2.2e-16	***
Time of incubation	3	1526.1	508.7	22.4685	1.826e-09	***
illumination conditions	1	2.4	2.4	0.1052	0.01	*
Temperature* time of incubation	18	2636.3	146.5	6.4688	5.822e-08	***
Time*light	3	129.6	43.2	1.9083	0.1397	
Residuals	52	52	1177.3	22.6		

SS: Sum square; MS: mean square.

variables that affect the germination of urediospores and the germ tube development. Thus initially, we tested each factor alone and its effect on the two response namely germination percentage and germ tube growth under light and darkness conditions using the analysis of variances (Tables 1 and 2). The summary of the analysis of variance indicates that all main effects and their interactions among the three factors (illuminations, conditions, temperature and time of incubation) were significant ($P < 0.05$). Spore germination was observed over a wider temperature range of 5 to 30°C. The percentage of germination gradually rose to a maximum and then abruptly declined and no germination occurred at 35°C. Furthermore, the effect of temperature on germ tube growth paralleled its effect on germination.

Mean percentages (and standard deviation) of urediospores that germinated at 20°C were 95 and 93%, respectively under dark and light conditions. The corresponding means of the longest germ tube at 20°C were 125 and 112 µm, respectively. The illumination conditions also affect the development of the urediospores. Hence, the darkness conditions seem to enhance significantly ($P < 0.05$) both the germination and germ tube growth. After 4 h of incubation, germination was significantly higher under darkness regime at 15, 20 and 25°C than under light conditions. The same observations were noted for the germ tube growth, where the length under dark condition attained 67, 5 µm after 4 h at 20°C; however, this was only of 59 µm under light condition. Furthermore, the experimental results were analyzed through response surface methodology (RSM) to obtain empirical model for the best response.

The coded and uncoded values of the test variables were used to optimize the variables namely temperature, time of incubation and illumination conditions. The germination of the urediospores and the development of germ tube depend on the individual effects of combinations of test variables and the results show a significant variation for each combination. The quadratic model was used to explain the mathematical relationship between the independent variables and the dependent response. The following regression equations for the two responses are Y1 and Y2.

$$Y1=43.57+ 10.32*A+19.79*B+ 5.04*C -42.59A^2 + 7.96B^2- 25.61 A^2*B \quad (2)$$

$$Y2=86.34+ 36.02*A+18.89*B+ 3.74*C -10.85A*B-71.45A^2 -5.70B^2--13.50A^2*B +6.99A*B \quad (3)$$

In which Y is the response variable and A, B, and C are the coded values of the independent variables, temperature, time of incubation and illumination conditions, respectively. The goodness of fit of the model was checked by the determination coefficient (R^2). In this case, the value of the R^2 is respectively 0.94 and 0.97 for Y1 and Y2. Hence, this values indicates that the sample variation of 94.0and 97.0% for germination and germ tube growth occurring were attributed to the independent variables and only 6 and 3% of the total variation cannot

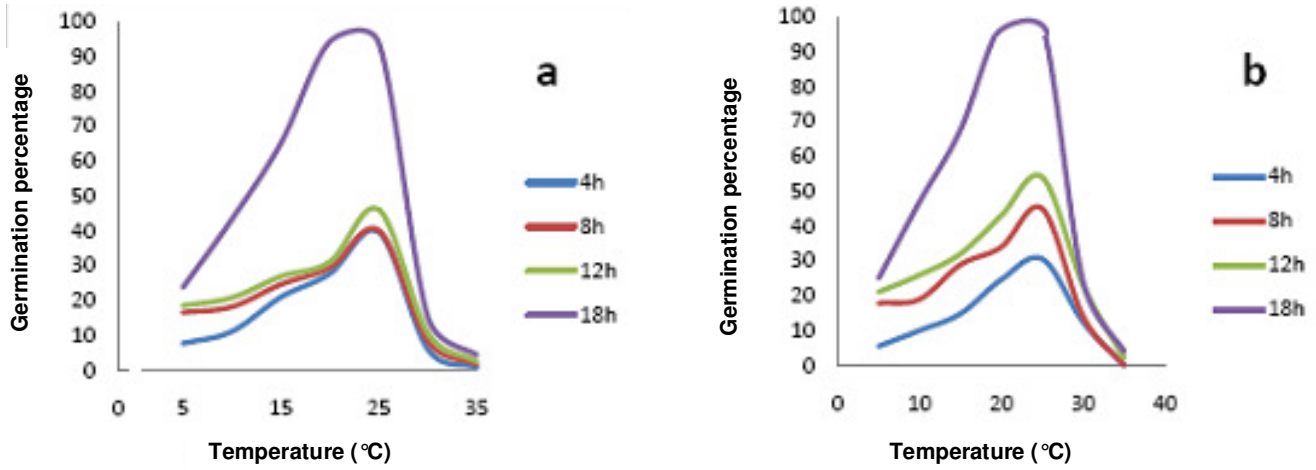

Figure 1. Percent germination of urediospores of *P.* coronata f.sp. *avenae* in both dark (a) and light (b) conditions.

be explained by the model.

The value of adjusted determination coefficient (adjusted R^2) is also high (0.92 and 0.96) for both Y1 and Y2 model, respectively which stresses the significance of the model. Furthermore, the high values of R (0.97 and 0.98). This correlation is also proven by the plot of predicted versus experimental values of germination and germ tube growth, as the entire points cluster around the diagonal line, which means that no significant violations of the model were found.

Each contour plot represents a number of combinations of two test variables with the other variable maintained at zero level. The maximum percentage of germination and germ tube development is indicated by the surface confined in the smallest curve of the contour plot. Analyzing the Figure 3, maximal germination and germ tube growth occurred when temperature were near 20°C after an incubation time of 16 h. Similarly, the maximum growth germ tube length occurred when temperature was near 22°C after an incubation time of 14 h. Thus, the corresponding values for the optimal germination and germ tube growth were 20°C and 8 h.

Response surface plot are more helpful in understanding the main and the interaction effects of these two test variables. Hence, the germination and germ tube development for different temperature and time of incubation can also be predicted from the respective response surface plots (Figure 3).

DISCUSSION

For large rust fungi, temperature plays a key role in spore germination, germ tube growth and eventual establishment of infection. Knowledge of the specific environmental requirements of *P. coronata* during this critical period should help to determine the threat of oat crop. Furthermore, the determinations of such environmental factors are important for the creation of favorable conditions for inoculation of plants in the greenhouse. These experiments attempting to elucidate the influence of environmental factors on urediospores germination and germ tube development under controlled conditions light or darkness conditions.

This study presents evidence that both the germination and germ tube growth of *P. coronata* are sensitive to the temperature and illuminations regimes. The range of optimum temperatures for the urediospores germination and germ tube growth were virtually the same. This temperature ranged from 5 to 30°C (Figures 1, 2). Approximately, 3 h were required for germination initiation to take place at 20 to 30°C. Temperature below 15°C delayed both the spore germination and germ tube development. The same results were also reported by many others authors, whose suggested that the *P. coronata* had a relatively broad range for germination of urediospores, which was at about 10 to 30°C (Chong and Kolmer, 1993; Chong, 2000; Chong et al., 2000).

Leu and Tu (1970) suggested that *P. coronata* behave similarly as others species of *P. graminis* on cereals which germinate well over a very broad range of temperature. Nevertheless, the same authors considered that different isolates of *P. graminis* could behave differently towards the environmental factors. Hence, isolates from Texas State germinated well over a much narrower temperature range comparatively to Mediterranean isolates. Similarly, the germ tube development was low under 15°C and over 25°C. This is similar to the findings of Osman-Ghani and Manners (1983) and Leonard and Martinelli (2005), whose noted that the germ tube growth for *P. coronata* sharply decreased under 15°C and which was also reported by Kolmer and Chong (1993). Moreover, our studies indicate that both germination and germ tube growth were influenced by the

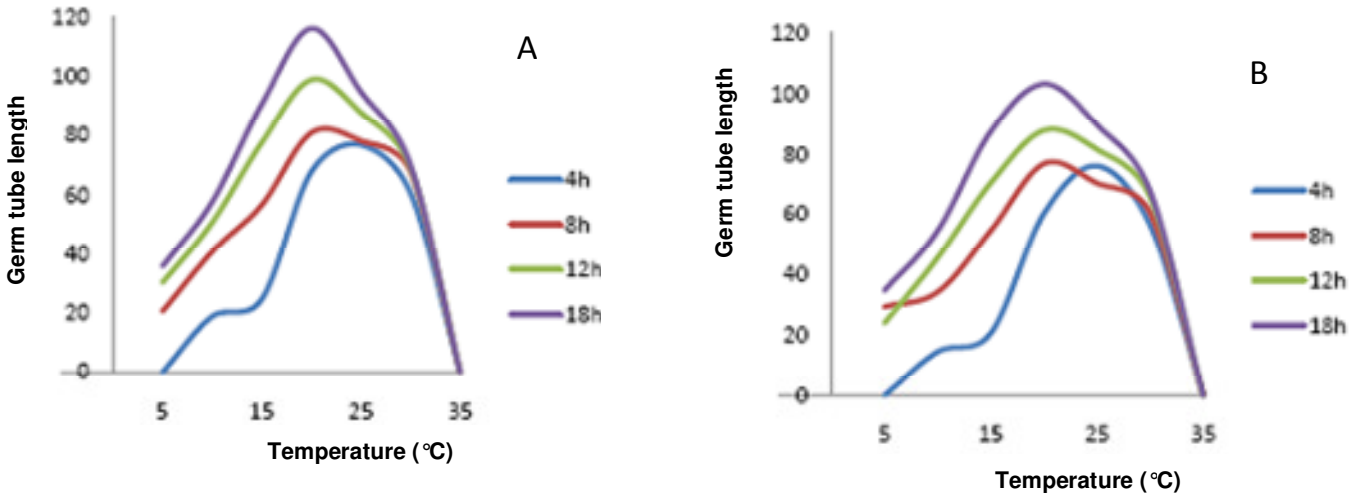

Figure 2. Germ tube length of urediospores of *P. coronata* f.sp. *avenae* in both dark (A) and ligh T (B) conditions.

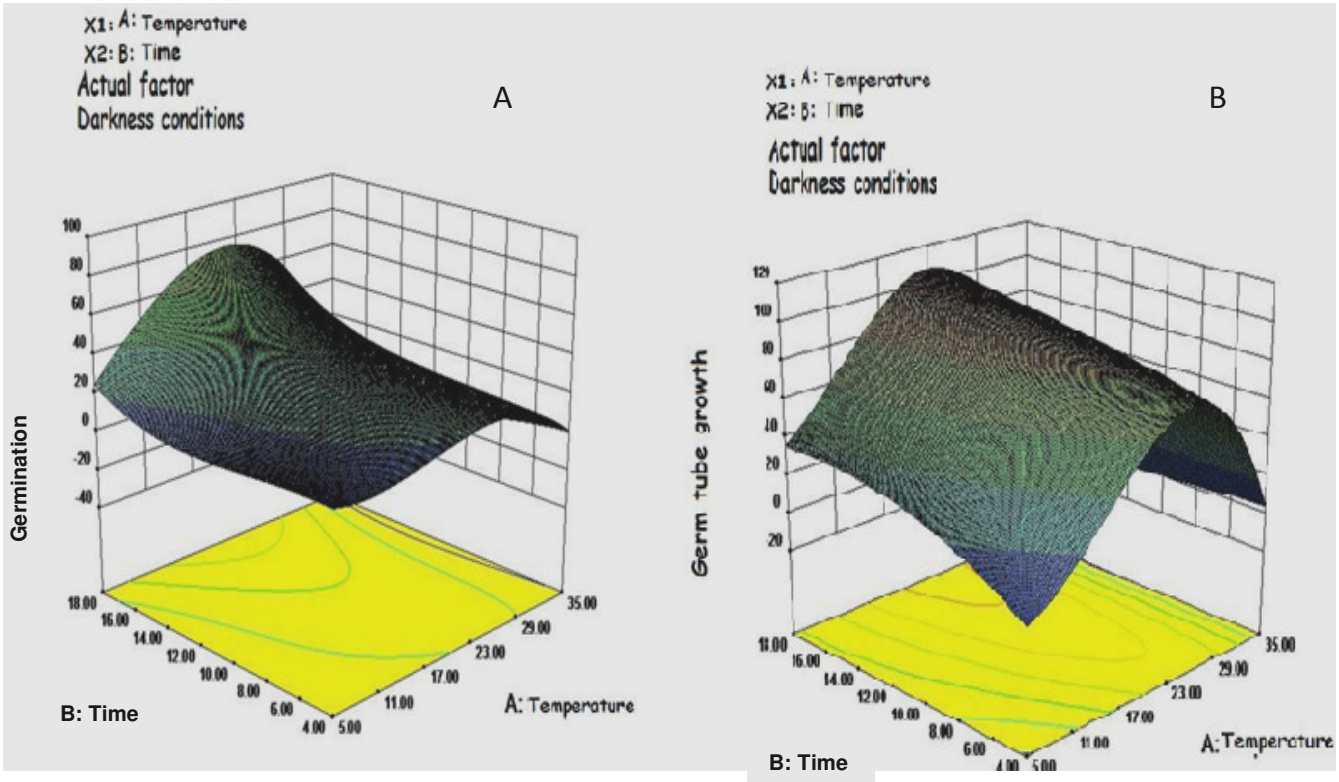

Figure 3. Response surface plot showing the effect of temperature, time of incubation on germination of urdiospores (a) and germ tube growth (b) in dark conditions.

illuminations conditions. Hence, the light had significantly reduced the germination and germ tube development. In fact, light inhibition of urediospores germination has been previously reported on others *Puccinia* species (Markell and Milus, 2008; Tapsoba and Wilson, 1997). Wilson (1994) suggested that fluorescent light, even at intensities less than 500 µE $M^{-2}S^{-1}$ was inhibitory to germination of *P. graminis*. Moreover, Givan and Bromfield (1964) and Tapsoba et al. (1997), observed that germination of urediospores was considerably better when under alternate light and darker than incubated in continuous light.

Table 3. Regression coefficients and their p-values for the quadratic linear regression model for predicting optimized responses (germination percentage and germ tube growth).

Germination response (Y1)			Germ tube growth (Y2)		
Factor	**b-Coefficient**	**p-value**	**Factor**	**b-Coefficient**	**p-value**
Intercept	43.57	< 0.0001*	Intercept	86.34	< 0.0001*
A-temperature	10.32	0.01483*	A- temperature	36.02	< 0.0001*
B-time of incubation	19.79	0.0362*	B- time of incubation	18.89	0.0003*
C-illuminations conditions	5.04	0.01089*	C- illuminations conditions	3.74	0.0230*
A*B	-4.03	0.2135	A*B	-10.85	< 0.0001*
A*C	0.70	0.7659	A*C	0.04	0.9688
B*C	0.56	0.7934	B*C	0.50	0.6456
A^2	-42.59	< 0.0001*	A^2	-71.45	< 0.0001*
B^2	7.96	0.0289*	B^2	-5.70	0.0030*
A*B*C	-0.69	0.8289	A*B*C	-0.38	0.8175
A^2*B	-25.61	< 0.0001*	A^2*B	-13.50	< 0.0001*
A^2*C	-3.95	0.3371	A^2*C	-3.54	0.0988
A*B^2	0.32	0.9503	A*B^2	6.99	0.0127*
B^2*C	-1.78	0.6088	B^2*C	-0.43	0.8079
Others statistics			**Others statistics**		
R^2	0.9374		R^2	0.9751	
Adj R^2	0.9265		Adj R^2	0.9658	
	SS	**Df**		**SS**	**Df**
Model	28375,19044	15	Model	57244,54629	15
Residual	5506,924014	40	Residual	1459,322107	40
Correlation total	33882,11446	55	Correlation total	58703,86839	55
F-value of model	13,740	-	F-value of model	10,460	-

Adj R^2: Adjusted R^2. *Coefficients with p-value greater than 0.05 quadratic model equations obtained by response surface methodology.

On the other hand, the results of analysis of variance for the model used for the germination and germ tube development of urediospores given in Table 3 demonstrate that the quadratic model was highly significant as is evident from the calculated *F* values of 13, 74 and 10, 46, respectively for germination and germ tube growth model and the very low probability value for the two models ($P < 0.0001$). Moreover, it was also observed from Table 3 that the coefficient for the linear and quadratic effects was highly significant when compared with many interactive effects.

Furthermore, both the R^2 and Adj R^2 were significantly high which indicate a high significance of the model (Akhnazarova and Kafarvo, 1982; Khuri and Cornell, 1987). On the other hand, a higher value of the correlation coefficient R (0.97 and 0.98) signifies an excellent correlation between the independent variables (Box et al., 1978).

The studies of the contour plots also reveal the maximum values which were 20°C and 16 h for germination and 22°C and 14 h for germ tube growth. The response surface plots also revealed the optimal values for both temperature and time of incubation which were 20°C after 8 h under darkness regime.

In fact, this differential ability in germination and germ tube growth could help the fungi to survive in broad temperature conditions. However, the validity of such hypothesis could be verified by using a large number of isolates from different geographic regions. In fact, variations in temperature requirements among isolates have also been encountered in others rust fungi (Tapsoba et al., 1997).

Hence, the response surface methodology proved to be a very useful and applicable tool for determining the characteristics of the variables tested in the germination and germ tube development, thereby, avoiding excessive analyses and offering generalized information on the influence of the independent parameters. Such approach, although satisfactory for the development a model of prediction of adequate temperature and time for germination and development of germ tube, may not reflect adequately the effect of environmental conditions

observed in the field.

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