ACTA MYCOLOGICA
VOL. XVI (1): 89—95
1980

Factors affecting growth and phenolic pigment production by Alternaria alternata (Fr.) Keissler

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Balwant Singh, K. L. Bajaj* and I. S. Bhatia: *Factors affecting growth and phenolic pigment production by Alternaria alternata* (Fr.) *Keissler.* Acta Mycol. 16 (1): 89-95, 1980.

The effect of factors such as composition of medium, pH, temperature and inoculation time on the amount of mycelial mass quantity and nature of phenolic pigments produced by the fungus *Alternaria alternata* (Fr.) Keissler has been studied. *Alternaria alternata* had maximum mycelial growth when the basal synthetic medium at pH 5.5, a temperature of 25° and incubation period of 12 days was used. The production of total phenolic pigment was maximum at temperature of 35° when the fungus was incubated for 9 days using basal synthetic medium. Addition of phenolic compouns such as tyrosine and gallic acid to the medium markedly increased the mycelial mass production but resulted in a suppression of phenolic pigment productien of the fungus while salicylic acid, cinnamic acid and catechol completely suppressed the growth.

INTRODUCTION

Alternaria alternata (Fr.) Keissler is a fungus causing a black core rot of mandarin oranges (Singh, Khanna 1960). The sulphur, phosphorus and nitrogen requirements of this fungus have been studied by earlier workers (Singh, Tandon 1967a, 1967b, 1970, 1971). Phenolic compounds are among the most important metabolites of fungi (Ollis 1961) because of their role in health and diseases of plants (Cruickshank, Perrin, 1964) and due to their inhibitory effect on fungal enzymes (Grossman 1967; Bhatia et al. 1972). The present investigation was undertaken to study the growth and production

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of phenolic pigments by *Alternaria alternata* under verying conditions. The effect of different phenolic compounds in the medium on the growth and phenolic production capacity of the fungus has also been investigated.

MATERIALS AND METHODS

The culture of the fungus *Alternaria alternata* (Fr.) Keissler obtained from the Department of Microbiology, Punjab Agricultural University, Ludhiana and grown on agar-agar slants in 50 ml Erlenmeyer flasks for 5-6 days. The mycelium was washed aseptically with sterilized water and macerated with 50 ml of sterilized distilled water. Two millilitros of this macerated mycelium was used to inoculate each flask. It was grown as a still culture at $25 \pm 1^{\circ}$ for a definite period in 250 ml Erlenmeyer flasks containing 50 ml of basal synthetic medium supplemented with 30 g of glucose per 1.

The different media of Hagam's (Armand, Thivend, 1965), Czapek's (Difco 1953), Chahal and Gray (1969) and Richard's (Ainsworth 1963) were used. Four different growth pHs, namely 3.5, 4.5, 5.5, and 6.5 were used. Incubation time varied from 6 to 18 days and observations were recorded at intervals of three days. The effect of four different temperatures, 15°, 25°, 35°, and 45°C was also studied. Tyrosine, cinnamic acid, salicylic acid, gallic acid and catechol at the concentration of 0.1% were added to the medium and their effects were studied.

A known weight of washed and dried mycelial mass was ground thoroughly in a pestle and mortar with acid washed sand. The ground material was transferred to an air tight glass stoppered flask (250 ml) to which 50 ml of ethyl acetate per gram of dried mycelium was added. The contents of the flask were shaken thoroughly for three hours and filtered through a sintered glass funnel. The residue was again treated with 3×20 ml of ethyl acetate per gram of mycelium for complete extraction. The extracts were combined and evaporated. The ethyl acetate extractable matter was dissolved in methanol and diluted to a known volume for the quantitative estimation of phenols by the colorimetric method of Swain and Hillis (1959) using Folin-Denis reagent and tannic acid as standard.

RESULTS AND DISCUSSION

Table 1 shows the average mycelial mass, total ethyl acetate extractable mather and phenolic pigments using different media. In this

Table 1

Effect of composition of medium on mycelial mass and pigment content of fungus (incubated at 25°C for 12 days at pH 5.5)

Medium	Average mycelial mass per flask (mg)	Total phenolic pigments per flask (mg)	Total phenolic pigments per g mycelial mass (mg)	Total acetate extractable matter (mg)
Czapek's	145.3 ± 8.0	0.36 ± 0.02	2.58 ± 0.08	13.10 ± 0.60
Basal synthetic	204.4 ± 11.6	0.41 ± 0.024	2.02 ± 0.09	14.60 ± 0.55
Richard's	179.0 ± 9.5	0.37 ± 0.021	2.06 ± 0.08	13.70 ± 0.45
Hagen's	166.0 ± 9.4	0.36 ± 0.02	2.16 ± 0.078	13.50 ± 0.42

context a distinction must be drawn between total phenolic pigments which is the quantity of pigments, synthesized in a fixed amount of medium and which is a true measure of the capacity of the fungus to produce such compounds and the phenolic pigments per g of mycelial mass which reflects the proportion of such compounds to the total mycelial mass. These data revealed that basal synthetic medium was best as far as mycelial mass, maximum extractable matter production and phenolic pigments were concerned. In Czapek's medium, the maximum amount of the total phenolic pigments per g of the mycelium was observed. Since the growth as well as total extractable matter were found to be maximum on the basal synthetic medium, only this medium was used in further studies.

Effect of temperature, pH and incubation period

Data in Table 2 show that production of mycelial mass in higher at 25° than at 15°. Temperatures higher than 25° resulted in lower mass

Table 2

Effect of temperature on mycelial mass and pigment producing capacity of fungus (incubated at pH 5.5 for 12 days)

Temperature	Average mycelial mass per flask (mg)	Total phenotic pigments per flask (mg)	Total phenotic pigments per g mycelial mass (mg)	Total ethyl acetate extractable matter (mg)
15°C	31.6 ± 36	0.08 ± 0.004	2.5 ±0.3	4.3 ± 0.5
25°C	214.0 ± 9.2	0.47 ± 0.03	2.2 ± 0.25	12.5 ± 1.5
35°C	170.0 ± 9.5	0.58 ± 0.05	3.4 ± 0.3	22.1 ± 2.5
45°C	no growth			

production and at 45° growth had ceased completely. The production of phenolic pigments per g mycelial mass and total phenolic pigments produced per flask were highest at 35°. Total extractable matter per g of mycelium first decreased than increased with higher temperature and this is probably due to the corresponding decrease in mycelial mass. The optimum pH for mycelial production and total extractable matter was found to be 5.5 and total phenolic pigments were maximum at pH 6.5 (Table 3). The content of phenolic pigments per g of mycelium

Table 3

Effect of pH of medium on mycelial mass and phenolic pigment producing capacity of fungus (incubated for 12 days at 25°C)

pH	Average mycelial mass per flask (mg)	Total phenolic pigments per flask (mg)	Total phenolic pigments per g (mg)	Total ethyl acetate extractable matter (mg)
3.5	100 ± 5.5	0.08 ± 0.004	0.783 ± 0.06	7.3 ± 0.35
4.5	133 ± 6.4	0.34 ± 0.02	2.59 ± 0.075	12.3 ± 0.5
5.5	210 ± 10.5	0.43 ± 0.025	2.03 ± 0.055	17.80 ± 0.07
6.5	140.87 ± 5.9	1.08 ± 0.06	1.27 ± 0.09	11.5 ± 0.04

was maximum at pH 4.5, and this was correlated with a decrease in mycelial mass. pH values lower than 6.5 considerably affected the contents of phenolics while pH higher than 5.5 decreased the content of extractable matter. A lag period of 3-4 days was observed followed by a rapid growth which continued up to 15 days after which autolysis of the mycelium started (Table 4). An increase in the extractable matter

Table 4

Variation of macelial mass and phenolic pigment producing capacity with age of fungus (incubated at pH 5.5 25°C)

Age of fungus in days	Average mycelial mass per flask (mg)	Total phenolic pigments per flask (mg)	Total phenolic pigments per g mycelial mass (mg)	Total ethyl acetate extractable matter (mg)
6	34 ± 4.2	0.27 ± 0.015	8.16 ± 0.05	9.5 ± 0.03
9	170 ± 9.2	0.39 ± 0.025	2.31 ± 0.06	15.7 ± 0.05
12	257 ± 18.5	0.39 ± 0.020	1.52 ± 0.06	16.4 ± 0.02
15	297 ± 14.6	0.36 ± 0.015	1.22 ± 0.06	14.4 ± 0.021
18	289 ± 12.5	0.34 ± 0.012	1.18 ± 0.07	12.8 ± 0.018

per flask was observed up to 12 days and was followed by a decrease in their content. The content of phenolic pigments increased up to 9 days, remained constant up to 12 days, then decreased. The proportion of phenolic pigments in the mycelial mass, however, showed a rapid decline between 6 and 9 days since during this period the rapid increase in the mycelium mass was accompanied by slight increase in phenolics. With the cessation of growth the decline in the proportion of phenolic pigments became less steep. Obviously the rate of synthesis of phenolic pigments was slow as compared to the rate of production of mycelium during the early period of incubation.

Effect of addition of different phenolic compounds

Results given in Table 5 show that addition of salicylic acid, cinnamic acid and catechol suppressed the growth completely, but gallic acid acted as a good stimulant for growth of the fungus. However, the total

Table 5

Effect of addition of phenolic compounds to the medium on mycelial mass and pigment producing capacity of fungus (incubated at pH 5.5 at 25°C in basal synthetic medium for 12 days)

Compound added	Mycelial mass flask (mg)	% increase in mycelial mass (on blank basis)	Total phenolic pigments per flask (mg)	Total phenolic pigments per g mycelial mass (mg)	Total ethyl acetate extractable matter (mg)
No (Blank)	201.7 ± 6.5	—	0.45 ± 0.04	2.212 ± 0.02	15.12 ± 0.15
Gallic acid	293.1 ± 7.5	45.3 ± 1.5	0.31 ± 0.035	1.044 ± 0.015	25.60 ± 0.55
Tyrosine	228.8 ± 4.8	13.2 ± 0.8	0.14 ± 0.01	0.614 ± 0.005	11.75 ± 0.18
Salicylic acid	no growth				
Cinnamic acid	no growth				
Catechol	no growth				

phenolic pigments per flask were lowered by the addition of tyrosine or gallic acid compared to the control experiment. In the presence of gallic acid, a higher content of extractable matter was obtained. Possibly, the presence of gallic acid in the incubation medium causes the formation of relatively complex compounds which are extractable by ethyl acetate but fail to react with Folin-Denis reagent (S w a i n, H i l l i s 1959). In case of *Phytophthora fragariae*, it has been shown that different phenolic compounds have different effect in retarding or increasing the growth (J a r v i s 1961). Sporulation of *Piricularia* isolates was generally depress-

ed by various phenolic compounds, however, it was stimulated in some isolates by caffeic acid, guaiacol and cinnamic acid (Narayanarao et al. 1972).

Acknowledgement

One of us (B.S.) thanks the Punjab Agricultural University authorities for the award of a merit scholarship during the course of studies.

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Czynniki wpływające na wzrost i wytwarzanie barwnika przez *Alternaria alternata*

Streszczenie

Autor badał wpływ składu pożywki, wartości pH, temperatury i czasu inokulacji na ilość masy grzybni i naturę barwników fenolowych wytwarzanych przez *Alternaria alternata* (Fr.) Keissler. Dodanie do pożywki związków fenolowych zwiększało rozwój grzybni, natomiast dodanie kwasu salicylowego, kwasu cynamonowego lub katecholu całkowicie go hamowało.