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Research Paper

Isolation of *Alternaria alternata* from Wheat Crop Spike Let

Microbiology

KEYWORDS : *Alternaria alternata,* black-point disease, Media, Mycelium, pH, Relative Humidity, Temperature, Wheat.

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ABSTRACT

The best growth of Alternaria alternata was observed on Malt Yeast agar medium with a colony diameter of 2cm after 4 days of incubation. The decline phase started from the 12th day of incubation and favored acidic as well as neutral pH range. At alkaline ph, hydrogen ion concentration had inhibitory effect on the growth and development of mycelia and pigmentation of the fungus. Best growth had been observed at a wide range of temperature between 20-35ºC and sporulation was observed at 28ºC, and 95 to 100% RH proved optimum.

INTRODUCTION

Alternaria, *Aspergillus* and *Fusarium* species can be found as pre-harvest fungal contaminants in wheat. This contamination affects the wheat milling industry due to low quality of wheat by products and the potential risk of mycotoxin contamination. Ripening ears of wheat are colonized by *A. alternata* soon after emergence and this specie is reported to be the most common sub epidermal fungus of wheat grains. *A. alternata* alone or with another fungus can cause a conspicuous black or brown discoloration of wheat kernels called black-point disease (Logrieco *et al.*, 2003).

The fungi commonly involved in the black point disease of wheat include *Alternaria alternata, Bipolaris sorokiniana, Cladosporium cladosporioides, Curvularia lunata* and *Fusarium* spp. (Fakir *et al.,* 1989; Dey *et al.,* 1992). Under the favorable conditions of disease development, the airborne inoculum's of the causal fungi infect the spikes and ultimately lead to the development of black point on the grains. However the characteristic discoloration of the kernels usually appears during the soft dough to hard dough stages of grain development (Talukder and Fakir, 1993; Ahmed *et al.,* 1994). The present study was undertaken to determine the prevalence of different fungi associated with floret lemma and developing grains and to assess the incidence of black point disease under different exposure periods of spikes to airborne inoculum's of the causal fungi. Wheat barley was surface disinfected for 2 minutes with 0.2% sodium hypochlorite solution and rinsed three times with sterile distilled water. From each sample, 40 grains were randomly selected and then put in Petri plates (90 mm diameter, 10 grains/dish) containing Sabouraud's dextrose agar with 5% chloramphenicol in duplicate. Petri plates were incubated at 25°C for 6 to 10 days. Culture was characterized and identified based on their morphological and microscopic characteristics using the keys of Pitt and Hockings and Raper and Fennel. Disease appears in mature stage of wheat, especially on late harvests. Sporulation of fungus becomes visible on spike lets, head and grains. *A. alternata* on spike lets and grains (especially in embryo) causes dark spots.

MATERIALS AND METHODS

Collection and isolation: Spike let portion were thoroughly washed in running tap water after which they were surface sterilized by submerging them in 75% ethanol for 2 min. The branch portions were further sterilized sequentially in 5.3% sodium hypochlorite solution for 5 min, and 75% ethanol for 0.5 min. spike let was divided into three segments and placed on potato dextrose agar (PDA) supplemented with 50 mg/l chloramphenicol to suppress bacterial growth. Branch portions were cut to expose their inner tissue and placed on the same medium. All the plates were incubated at 28°C for up to 3 weeks. Emerging fungi were transferred to fresh PDA plates, incubated for 1 week and periodically checked for purity.

Identification: Slide culture technique was adopted for the identification of *Alternaria alternata* isolates of spike let after 150 days sowing wheat morphological characteristics was prepared by keeping thin cotton pad, a wet filter paper and a slide inside a sterilized petriplate. Saboraud dextrose agar medium was prepared and poured on sterilized glass plate in the form of thin film. After solidification, this film was cut into small cubes with flamed scalpel. These cubes were placed on slide inside moist chamber and inoculated with fungal spores separately. These slide culture were incubated at 28 ±1ºC in a BOD incubator, after sporulation the slides were stained with cotton blue mounted in lacto phenol and were observed for identification. For permanent mounts, stained fungal structure was mounted using DPX. Fungal identification was done on the basis of morphotaxonomic characters. Careful microscopic examinations were carried out to ensure that the culture were not mixed.

Virulence (Pathogenicity) of the isolates: The pathogen city of purified isolates of *A. alternata* was tested and it was proved by Koch's Postulates conducted on the spike let of Wheat (Elwakil el al,Jain et al.,2005).The plants were raised in pots under glass house conditions The conidial suspension was prepared sterile distilled water from 9 day old PDA cultures of the isolates of *A. alternata*.the spore suspension were sprayed on the wheat 150 days old crop Water congestion was provided to the plants both 24 h prior and after inoculation by covering the plants with moist polythene bag. The inoculation was done on cool evening hours. The Plants sprayed with sterile distilled water served as control. Inoculated plants were maintained in glass house condition. The severe symptoms were observed on14days after inoculation.The symptoms were observed and compared with the original symptoms. The fungus was reisolated from artificially inoculated wheat spike let.

Evaluation of mycelia growth and spore production of *Alternaria alternate*: Homogenous fungal culture was obtained by spreading 0.1ml spore suspension, containing 10^6 spores per ml in water with 0.05% Tween 80, evenly onto PDA Medium in Petri dishes were incubated at 28± 1º C in the dark. After two days mycelium plugs were removed with a sterile cork borer and transferred to Petri dishes with either Potato Dextrose Agar, Potato Carrot Agar, Malt Extract Peptone, Malt YEAST AGAR,SDA,COON'S, Czapek's, Richards and casein agar media with different potentials Measurements of the mycelia growth were taken after three and six days of incubation at 28±1ºC. The diameter of the colonies was estimated by calculating the mean of two perpendicular measurements. The sporulation rate was assessed after six days. The Petri dishes were rinsed with 1 ml of tween 80 and the conidia were scraped off carefully with a spatula. The spore con-

centration was determined with a haemocytometer and the viability of the conidia was examined after incubation for 24hours at 28±1º Celsius on SDA.

Growth pattern of *Alternaria alternata*: Conical flasks containing 25 ml SD Broth each were inoculated with 1ml of conidial suspension of *Alternaria alternata* and were incubated at 28th ±1ºC degree Celsius. The dry weights of mycelia were taken from 2 to 15 days of incubation.

Determination of an effective growth of *alternaria alternata*: 40 ml of SDA medium of ph gradient from 2 to 12 was prepared and sterilized and autoclaved medium was poured in presterilized Petri plates after solidification of the medium, plates were inoculated by spot inoculation with *Alternaria alternata.* The inoculated plates were then maintained at 28 ±1 degree Celsius. The colony diameter and characteristics were observed daily after an incubation period of 48 hours.

Effect of different temperature on the growth of *Alternaria alternata*: The method described by Sandhu (1995) was followed. Inoculated Petri plates were incubated at different temperature viz, 25, 28,30,35,40 and 45ºC and observation were recorded 7 days of incubation.

Effect of different relative humidity on the growth of *Alternaria alternata*: In the investigation, effect of different relative humidity's on the growth and sporulation of *Alternaria alternata* was observed on solidified malt agar medium supplemented with 1% yeast extract. Inoculated petriplates were incubated at different relative humidity *viz.* 53, 75, 85, 95 and 100% (Sandhu, 1995). Observation were recorded after 7days of incubation.

RESULT AND DISCUSSION:

Detection of *Alternaria alternata*

In the laboratory this fungus was found capable to grow on almost all the media tested with different growth patterns. This experiment was conducted for selecting the best suitable medium for the growth of *Alternaria alternata.* The growth on different media was measured by taking the diameter of the colony after 4 days incubation, because radial increase in colony is the only reliable characteristics for measuring growth on different solid media, as it can be measured repeatedly (Vaidya, 1995). The best growth was observed on Malt Yeast Agar medium with a colony diameter of 2 cm after 4 days of incubation (Table -1). In a similar study, Vaidya (1995) has reported that yeast extract in the medium contains some unknown substances, which are required for the optimal growth of certain fungi.

Growth pattern of *Alternaria alternata*: Growth is considered as an irreversible increase in the mass /volume of an organism that occurs after a given period of incubation in nature or in laboratory. Growth curve best was done to get the juvenile growth stage of fungal mycelia It is necessary to consider vegetable growth in fungi separately as it exhibits different mode or patterns of growth. The results presented in table-2 revealed that the stationary phase of growth of *Alternaria alternate* occurred from 7th with the decline phase starting from the 12th day of incubation.

Effects of hydrogen ion concentration or ph on the growth of Alternaria alternata Environmental factors greatly influence the behavior of fungi. Under natural conditions fungi obtain all their requirements from the host substratum for the synthesis of their cell constituents and also for the operation of their life processes. The biological studies pathogens have a special importance in understanding their behavior in the development of *Alternaria alternata.* Favors acidic, poor mycelia development was observed on alkaline medium. At alkaline ph; hydrogen ion concentration had inhibitory effect on the growth and development of mycelia and pigmentation of the fungus. Since change in the ph of a medium may also change its composition if it contains weakly ionized constituents, the physiological effects of dissociated and undissociated species were quite different. Most of the fungi grow within the ph range 4-8, many fungi grow over a wider range, and a few have been reported to have a narrower range (Lilly and Barnett, 1951and tendon, 1961).

Effect of different temperatures on the growth of *alternaria alternata*: Temperature has been considered for a long time to be one of the important factors affecting the natural activity of parasitic fungi. The importance of favorable temperature for the optimal growth of *Alternaria alternata* fungi has been mentioned by Steinhaus (1949).Much information is available on the effect of temperature on growth of *Alternaria alternata* fungi in vitro (Muller-Kogler, 1965). Generally, the limit for growth ranges between 5-35˚C and the optima fall between 20 and 30˚C (Robert and Yendol, 1971 and Ferron, 1978). They also studied the influence of temperature and relative humidity on muscardian fungi and stated that the rapidity of mycelia development, and thereby, the rapidity of the infection, depend on temperature, It was observed that *Alternaria alternata* could grow at a wide range of temperature between 20-35ºC,The best growth and sporulation of the fungus, were observed at 28ºC and 30ºC, as exhibited by colony diameter i.e.2.6 and 2.5 cm and sporulation respectively. At 25ºC the colony diameter was 2.3 cm and sporulation was good. Colony characteristics were similar, except that much flosses growth and pale droplets were visible in sprouting colony

Effect of different levels of relative humidity on growth of *Alternaria alternata*: It is not only the temperature, which influences the growth and sporulation of fungi but also many factors, like light, chemical composition of the substrate, micro and macro environmental humidity and the interaction of these factors in the ecological complex that influence the development *Alternaria alternata* (Yendol and Hamlen 1973). Humidity plays a very important role in sporulation of the pathogen. The present isolate if *Alternaria alternata* was capable to grow and sporulate at different levels of relative humidity. The fungus attained its best growth and sporulation at 95 and 100 % RH, with colony diameter measuring 2.6 and 2.9 cm, respectively. At 53% RH, the colony diameter was 1.6 cm with moderate sporulation and at 75 and 85% RH it was 1.8 and 2.0 cms respectively with good sporulation. The colony characteristics at alternative humidity were similar except that the fungal growth was highly floccose and powdery in the sporulating fungus at 95and 100%RH.With the increase in relative humidity, the growth and sporulation of the fungus increased. Although 100%RH supported excellent sporulation with spore count ca.1.46×10^6 spores/ml, it was also good at lower values of relative humidity.

The minimum time required for sporulation in saturated atmosphere was found to be 3 days. Lower humidity levels had some sort of retarding effect on the growth and sporulation similar results were obtained by Veen (1968) and Phadke (1983). At 100%RH spores developed within 4 days but they required 5 days or longer period at 92.5-98%RH. *Alternaria alternata* sporulated with relative humidity as low as 53% which proved its superiority over other isolates.

Table -1: Effect of different media on the growth of *Alternaria alternata*

No	Name of medium	Colony diameter (cms)after 4 days of Incubation
1	SDA	1.8
2	Malt Yeast agar	2.0
3	PDA	1.7
4	PCA	1.8

5	Richard's	1.4
6	Casein Agar	1.2
7	Coon's	1.1
8	Czapek's	1.9
9	MEP	1.3

Table-2: Growth pattern of *Alternaria alternata*

Days of incubation	Weight of filter paper (g)	Weight of filter paper and mycelia	Weight of mycelia
	A	B	C=B-A
1	0.453	0.598	0.145
2	0.388	0.550	0.162
3	0.435	0.618	0.183
4	1.107	1.292	0.185
5	0.996	1.216	0.220
6	0.961	1.236	0.275
7	1.014	1.330	0.316
8	0.650	1.130	0.480
9	0.971	1.448	0.477
10	0.812	1.288	0.476
11	0.558	1.031	0.473
12	0.817	1.203	0.386
13	0.567	0.852	0.285
14	0.616	0.887	0.271

Table-3: Effect of pH on the growth of *Alternaria alternata*

S. No	pH	Colony diameter (cms)	Colony Characteristics
1	2	NA	No Growth
2	3	NA	No Growth
3	4	1.9	Thick, brown to black
4	5	1.8	Thick , brown to black
5	6	2.1	Thick ,brown to velvet black
6	7	2.0	Thick, brown to black
7	8	1.7	Light ,brown to black
8	9	1.8	Brown to Black
9	10	1.7	Brown to Black
10	11	1.5	Brown to Black
11	12	1.3	Brown to Black

Table-4: Effect of different temperatures on the growth of *Alternaria alternata*

S. No	Temp.	Colony Diameter (cm)	Sporulation	Spore count/ml	Colony Characteristic
1	5°C	00	No Growth	NIL	Inoculums started
2	10°C	0.2	No Growth	NIL	Inoculums started rooting with few vegetative brown mycelia growth
3	15°C	0.3	No Growth	NIL	Inoculums started rooting with vegetative brown mycelia growth
4	20°C	1.8	Poor	3020	Color is initially brown to black with good mycelia growth
5	25°C	2.3	Good	5300	Thick brown to black colony,
6	28°C	2.6	Excellent	6500	Thick brown to black colony,
7	30°C	2.5	Good	5500	Brown to Black colony
8	35°C	0.4	Poor	1050	Thin black colony
9	40°C	0.1	Poor	1000	Poor mycelia growth
10	45°C	00	No Growth	NIL	No growth

Table -5: Effect of different levels of relative –humidity on the growth of *Alternaria alternata*

S. No	Percent Relative Humidity	Colony diameter (cm)	Sporulation	Spore count (ml)	Colony Characteristics
1	53	1.6	Moderate	1.3×10^4	Colony black
2	75	1.8	Good	0.95×10^5	Black
3	80	2.0	Good	1.4×10^5	Thick Black
4	95	2.1	Excellent	1.5×10^5	Thick black
5	100	1.9	Good	1.45×10^5	Thick Black

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