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Research Article

EFFECT OF SOME ENVIRONMENTAL FACTORS ON GROWTH AND SPORULATION OF *CERCOSPORA BETICOLA* SACC., THE CAUSAL AGENT OF LEAF SPOT DISEASE OF SPINACH BEET

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ABSTRACT

The present study evaluates the effect of some environmental factors on growth and sporulation of two isolates of *Cercospora beticola in vitro*. *C. beticola* isolates (A & B), were obtained from infected spinach leaves collected from two farms at Glen valley and Otse respectively in Gaborone, Botswana. The two isolates were used to study phenotypic variations amongst isolates of this pathogen, effect of six different media (potato dextrose agar, yeast extract agar, Czapekdox agar, spinach beet decoction agar, corn meal agar and carrot decoction agar) effect of temperatures ranging from 10^{o} C – 30^{o} C., effect of pH (pH 3 – 9) and effect of light regime (normal day light and dark) on growth and sporulation of *C. beticola*. Parameters used were diameter of colony in agar media for the period of 30 days at five days interval and dry weight of mycelia in liquid media and number of conidia produced per ml of conidia suspension obtained from the culture. The data obtained was subjected to Microsoft excel for two- way analysis of variance (ANOVA), and mean separation tests were carried out using the Least Significant Difference (LSD) test at p= 0.05. The two isolates, A & B of *C.beticola* did not exhibit any difference in morphological characteristics on different media. There was significant difference between growth and sporulation on different media with the best growth on spinach beet decoction agar and carrot decoction agar, and the best sporulation on spinach beet decoction agar for both isolates of *C. beticola* obtained. In liquid media, carrot decoction supported the highest fungal growth and sporulation for two isolates of *C. beticola*. There was significant difference between growths at different temperature. The best growth and sporulation were observed at 25^{o}C and total inhibition above 30^{o}C. The pathogen grew best on pH 6 and least growth occurred at pH 9 and pH 3.

Keywords: *C.beticola, Spinach Beet, Media, Temperature, pH, Light Regime, Growth, Sporulation*

INTRODUCTION

Cercospora leaf spot of spinach beet caused by *Cercospora beticola* Sacc., is considered to be the most economically important foliar disease of spinach beet in Botswana, reducing quality, quantity and marketability of spinach leaves. Spinach beet (*Beta vulgaris var.cicla* (L.) W.D.J. Koch, also known as Swiss chard is a leafy green vegetable and grown for its dark green leaves. The most common varieties of spinach beet are Ford hook Giant, Rhubarb Chard and Lucullus. Ford hook Giant and Lucullus varieties of spinach beet are usually grown in Botswana and heavily infected with *Cercospora beticola*. Other major foliar diseases of spinach beet include blue mold (*Peronospora effusa*), white rust (*Albugo occidentalis*), anthracnose (*Colletotrichum spinacicola* and *C. spinaciae*) and Cladosporium leaf spot (*Cladosporium macrocarpum*) which have been reported elsewhere but not of significantly important in Botswana (Correll *et al.,* 1994).The ability of *C.beticola* to infect wide range of plants shows that it is an aggressive pathogen which can destroy many different crops growing within the same area. Recent study revealed that *C. beticola* could infect sun flower during artificial inoculation experiments (Weiland and Koch, 2004). During periods of warm temperatures and high humidity or leaf wetness, *C. beticola* on spinach beet form tan necrotic spots on lower leaves which will turn gray and become blighted all over the leaf and thus lowering quality or making the spinach leaves unmarketable. To authors knowledge there is no information available on the pathogen and the disease in Botswana. Therefore, a research project was undertaken to study the different aspects of this important disease and its causal agent with

ultimate goal to manage the disease. This paper however, reports the effect of some environmental factors on growth and sporulation of *C. beticola*, the causal agent of spinach beet in Botswana.

MATERIALS AND METHODS

Isolation and Identification of Cercospora beticola

Fresh infected spinach beet leaves collected from randomly selected farmers fields at two different locations, Glen valley and Otse in Gaborone, Botswana were observed through transmitted light under the stereo microscope and preserved at 4^{o}C for subsequent use. Some of leaves with a lot of lesions were kept in a moist chamber to induce sporulation for isolating and characterizing the pathogen. The pathogen were then isolated from infected leaves pieces and cultured on the Potato dextrose agar. The plates containing infected tissue were incubated at 25^{o}C. These were examined daily to check growth of fungus around the tissue pieces. Once the growth of the fungus has occurred it was sub-cultured on other sterile PDA plates and incubated at 25^{o}C. The cultures obtained were stored at 4^{o} C for subsequent use.

The symptoms of the disease and the characteristics of conidia both from the infected leaves and the cultures were examined. After consulting references Chupp (1953), Groenewald *et al.*, (2006) and Crous *et al.*, (2006) the pathogen isolated was identified to be *Cercospora beticola*. The colony that was identified as *C. beticola* was sub cultured into other prepared sterile PDA plates and cultures thus obtained were kept at low temperature for later use in the experiment.

Preparation of Media for the Experiment

For the morphological and cultural characterization, isolates of *C. beticola* were cultured on six different media, potato dextrose agar, yeast extract agar, Czapekdox agar, corn meal agar, spinach beet decoction agar and carrot decoction agar. The former four media were prepared as per instructions given by the manufacturer. For preparation of spinach beet decoction medium, 200 g of fresh spinach beet leaves was chopped into small pieces and boiled in 500 ml bottle containing 500 ml of distilled water for 15 minutes, and then the extract was filtered through a muslin cloth into 1000 ml bottle. Twenty gram dextrose and fifteen gram powder agar were added to the filtrate and made into one liter. The mixture was dissolved in hot plate using magnetic stir and dispensed into 100 ml bottles which were autoclaved at 121^{o}C for 15 minutes. Carrot decoction media was similarly prepared.

Growth on Solid Media

The growth and sporulation of *C. beticola* were studied in six different media as mentioned earlier. All the media were sterilized in autoclave at 121^{o} C for 15 minutes. 15 ml of each medium was poured into 90 mm diameter petri plates. 5mm mycelia discs were cut from the margins of 14 day old cultures of *C. beticola*. One disc was placed aseptically onto each sterile petri plate with each sterile medium. Each treatment was replicated thrice. Colony diameter of the pathogen on each medium was measured in millimeters every five days interval for one month and the averages were taken. After 30 days of incubation, spore suspensions were prepared by slowly adding about 10ml of sterile distil water into the each culture plate with each sterile medium. Sterile needle was used to scrap the surface of the culture medium gently to favor detachment of conidia, and transferred the suspension into tubes, and the tubes were then shaken vigorously to break the spore clumps. An aliquot of this suspension was taken and the number of conidia per ml was quantified with the help of haemocytometer. The colony color and surface elevation were observed at the end of the incubation period. The data on radial growth was analyzed statistically. The type of solid medium which had best fungal growth was chosen and used to study the effect of other environmental factors on growth and sporulation of *C. beticola* isolates.

Growth in Liquid Media

The growth of the pathogen was measured in five liquid media (Carrot decoction, Spinach beet decoction, Potato dextrose, Corn meal and Czapekdox). Twenty ml of each medium was poured in each of the 100 ml flasks. These flasks were sterilized in autoclave at 121^{o}C for 15 minutes. Flasks when cooled were inoculated with five mm mycelium discs cut from the 14 days pure culture of two isolates of *C.beticola* and incubated on a rotary shaker at 25^{o}C. Each treatment was replicated thrice. After 20 days, the mycelium growth was harvested and filtered through Whatman No. 42 filter paper, which were previously

dried to a constant weight in hot air oven at 60°C. The mycelial mat on the filter paper was thoroughly washed with distilled water to leach out any salts associated with the mycelium. Subsequently, the filter papers along with the mycelial mat was dried to a constant weight, cooled in desicator and weighed on an electronic balance. Weight of *C. beticola* mycelium mat was calculated by subtracting the weight of dried filter paper from the weight of dried filter paper with mycelia mat. The data was analyzed statistically.

Effect of Temperature on Growth and Sporulation of C. beticola

Five millimeter mycelia disc obtained as mentioned earlier was transferred onto each spinach beet agar medium. These plates were incubated at different temperatures ranging from 5°C -35°C at an interval of 5°C. The plates were replicated thrice at each temperature. Colony diameters were measured in millimeters every five days interval for thirty days, and the averages taken for each temperature. Temperature which has best fungal growth was selected and used to study the effect of pH. After 30 days of incubation spore suspensions were prepared as mentioned earlier and sporulation was quantified with the help of haemocytometer at each temperature.

Effect of pH on Growth and Sporulation of C. beticola

To test the effect of pH on growth and sporulation of *C. beticola* isolates, mycelia discs of 5-mm diameter obtained as mentioned before was aseptically placed onto each of the sterile petri-plates with the spinach beet agar medium adjusted with the help of hydrochloric acid and sodium hydroxide at different pH ranging from pH 3- pH 9 by using pH meter. The plates were incubated at 25°C. The plates were replicated thrice. The colony diameters were measured and the averages were taken for 30 days at 5 days interval. After 30 days spore suspensions were prepared and sporulation was quantified with the help of haemocytometer in each pH as mentioned before.

Effect of Light Regime on Growth and Sporulation of C. beticola

To test for the effect of light regime on growth and sporulation of *C. beticola* isolates 5 mm mycelium disc was cut from 14 day old culture of *C. beticola* using cork borer and aseptically placed on spinach medium adjusted to pH 6 and, the medium was incubated at 25°C. Some plates were put in dark while others were placed in normal day light. The diameter was measured and averages were taken at 5 days interval for 30 days. After 30 days spore suspension prepared as mentioned earlier and sporulation was quantified with the help of haemocytometer in petri plates in the dark and light.

RESULTS AND DISSCUSIONS

The symptoms of the leaf spot disease were characterized by production of small brown spots on leaves, in the beginning which turns to dark brown spots. The lesion varied in size, some had diameter ranging from 0.4 mm to 0.5 mm, other leaves exhibited larger spot which were spreading through the leaf and blighted the leaf. Spots with circular or irregular margin, appearing grayish in the center. Closely situated spots coalesced and formed large necrotic patches. Later they become papery thin and spots had straw coloured center and brown margin sometimes symptoms appeared on petiole as brown spots. *Cercospora beticola* formed uniformly compact colonies on potato dextrose agar. The colonies generally appeared black at the edges with greyish centre. *C. beticola* grew very slowly on potato dextrose agar medium attaining a maximum growth of 60-75 mm after 30 days of incubation at 25° C. Two isolates A and B, did not differ in phenotypic (morphology and colony characteristics) variations.

Results on the growth and sporulation of two isolates of *C. beticola* presented in Table 1indicated that *C.beticola* isolates A & B grew best on spinach beet decoction agar (isolate A: 81 mm, isolate B: 82 mm). These could have resulted from the presence of the nutrients from the host plant that promoted growth of the pathogen. Thiswas followed by carrot decoction agar, potato dextrose agar, corn meal agar, czapekdox agar and yeast extract agar. Minimum growth was observed on yeast extract agar (isolate A: 34mm, isolate B: 37.1mm), suggesting that media lack nutrients that are essential for growth of *Cercospora beticola*. The isolates A and B did not show significant differences among them in their growth on all six media. The result showed that there was steady increase in growth from 5 days to 25 days in all six media.

The sporulation was excellent in spinach decoction agar and carrot decoction agar; good on potato dextrose agar and poor on corn meal agar for both isolates of *C. beticola*A & B. No sporulation occurred either on czapekdox agar or yeast extract agar. Both the media (spinach decoction agar and carrot decoction agar) contained nutrients which favored sporulation. Alabi *et al.,* (1971) reported that the *Cercospora* species are known to grow and sporulate better on media which contain host and vegetable extracts.

Study of morphological characteristics of *C. beticola* isolates on different media confirmed that they have an identical texture and colour of mycelium beneath the medium, but vary in colour and width of the borderline. Kumara and Rawal (2008) suggested that wide range of media are used for isolation of different groups of fungi that influence the vegetative growth and colony morphology, pigmentation and sporulation depending upon the composition of specific culture medium. Isolates of *C. beticola* formed two types of mycelia on PDA, whitish and gray colour. Significant variation in mycelium border line, colour and width was observed on the surfaces of the different media as well. Good mycelia growth, ash coloured colonies with smooth margin was observed on spinach beet decoction agar and, flat, fluffy colonies observed on Carrot decoction agar.

Table1: Effect of different types of media on growth (colony diameter in mm) of two isolates (A & B) of *C. beticola*during 30 days of incubation at 25°C

Media	Average Colony diameter in mm at 5 days interval											
	5 days		**10 days**		**15 days**		**20 days**		**25 days**		**30 days**	
	A	B	**A**	B	**A**	B	**A**	B	**A**	B	**A**	B
CDA **bcd**	14.8	14.6	33.3	33.5	54.3	56.7	69.7	70.3	79.2	81.1	79.0	80.1
SBDA **bcd**	15.4	15	38.3	39.7	56.7	58.3	70	71.3	81.2	82.7	81.0	82.0
PDA **bc**	12.7	14	27.3	28.6	51.7	52.3	66.7	66.9	74.3	74.2	74.1	74
YEA **a**	6.7	7.3	13.7	14.1	18.7	19.3	29.1	30.7	34.0	37.3	34.0	37.1
CDA **ac**	6.0	6.7	12.3	13.7	24.9	24.6	40.0	41.7	45.0	46.7	44.9	46
CMA **ac**	9.3	8	23	22.3	41.5	40.7	50.7	49.8	56.3	51	56.3	51

In a column media followed by the same letters are not significantly (*P = 0.05*) different from each other according to Least Significant test. CDA – Carrot decoction agar, SBDA- spinach beet decoction agar, PDA – potato dextrose agar, YEA – yeast extract agar, CDA – Czapekdox agar, CMA – cornmeal agar.

No significant and stable difference was detected in growth dynamics and morphology of the mycelia for isolates of A and B developed on the same nutrition medium. This is in disagreement with Groenewald (2007) who reported that the isolates of *C. beticola* originating from the same spot significantly vary in morphology, regardless the nutrition medium they were developed on. No clear pattern of the morphology associated with the geographic origin, was determined for both isolates of *C. beticola* due to a high homology of congeneric species. The isolates of *Cercospora beticola* obtained were not significantly different in the growth on the different type of media compared to each other according to two- way ANOVA at, F= 0.001 and P= 0.005, but the growth of mycelia in all different types media were significantly different from each other.

The results on the effect of different types of liquid media on the growth and sporulation of *C. beticola* presented in table 2 showed that the pathogen grew best on carrot decoction (Isolate A: 1.4244 mg, Isolate B: 1.2369 mg) which was significantly superior to all other liquid media. This was followed by spinach beet decoction, potato dextrose and Czapekdox. Least mycelia weight was obtained in corn meal broth (Isolate A: 0.2412 mg, Isolate B: 0.2558 mg).The growth of mycelia in all liquid media was significantly different from each other butNo striking differences were noted in the mean dry mycelia weight on the two isolates of *C. beticola*. But no striking differences were noted in the mean dry mycelia weight on the two isolates of *C. beticola.* Mallapa (2007) suggested the determination of dry mycelia weight as the best method for precise growth because as it is not possible to consider the amount of submerged mycelium in

the radial measurements. A good sporulation was obtained on carrot decoction followed by spinach beet decoction, and sparse sporulation on PDA and no sporulation occurred either on Czapekdox or corn meal. The ability of the fungus to grow best in carrot decoction indicates the presence of certain nutrients and vitamins required by the pathogen. Sharma and Pandey (2010) suggested that carrot decoction agar was the best medium for sporulation to many species of *Cercospora* from different hosts.

Table 2: Effect of different liquid media on growth (dry mycelia weight in g) and sporulation of two isolates (A &B) of *Cercosporabeticola* Sacc., after 20 days of incubation at 25°C

Liquid media	Mean dry mycelia weight in g		Sporulation/ml
	Isolate A	Isolate B	
Spinach beet decoction**bcd**	1.0369	0.9757	++
Potato dextrose **bc**	0.8906	0.8769	+
Corn meal **b**	0.2412	0.2558	_
Czapekdox**a**	0.4378	0.3852	_

+++:> 1.5 x 10^5spores/ml, ++: 1.5 x 10^5 – 1 x 10^5spores/ml, +: < 1 x 10^5 spores/ml, - Nil. Means in a column followed by the common letters are not significantly different, p=0.05, LSD test.

The result on the effect of different temperatures on growth and sporulation of isolates of *C.beticola* presented in Table 3 showed that growth at 25˚C was significantly different from growth at the rest of the other temperatures. Growth at 5°C, 10˚C, 15˚C, 30˚C and 35˚C were not significantly different at p=0.005.There was steady increase in growth from 15^0-25^0C. The best growth and maximum sporulation of the pathogen occurred at 25^0C (isolate A: 80.9 mm, isolate B: 79.7 mm). Lowest growth without any sporulation was observed at 15^0C (isolate A: 18.9 mm, isolate B: 20.1 mm), and the growth was completely inhibited at 5^0 C, 10^0 C and 35^0C, suggesting that very high temperature and low temperature are not suitable for disease development as they results in rapid drying of mycelia. Sparse sporulation was observed at 20^0C and the temperatures at 35^0C, 15^0C, 10^0C and 5^0C inhibited sporulation of *C. beticola.* Each fungus has its temperature range for the growth and sporulation. The present study revealed that temperature ideal for best fungal growth was also good for induction of sporulation, as also mentioned by Griffin (1994).

Table 3: Effect of different temperature on growth and sporulation of two isolates (A &B) of *Cercosporabeticola* Sacc., on spinach decoction agar during 30 days of incubation

Temperature	Average Colony mean diameter in mm at 5 days interval												Sporulation	
	5 days		10 days		15 days		20 days		25 days		30 days			
	A	B	**A**	B	**A**	B	**A**	B	**A**	B	**A**	B	**A**	B
5^0Ca	5	5	5	5	5	5	5	5	5	4.9	4.9	4.9	-	-
10^0C a	5	5	5.4	6.1	6.4	6.1	5.8	7	5.8	7.1	5	7.1	-	-
15^0C a	6.7	6.4	11.1	11.8	15.9	15.5	17.4	17.8	18.9	20.1	18.9	20.1	-	-
20^0C b	9.7	10.1	19.8	20.8	35.7	34.8	47	46.7	68	67.3	68	67.3	++	++
25^0C bc	12.4	12.3	31.3	36.7	54.1	53.5	67.6	63.7	80.9	79.7	80.9	79.7	+++	+++
30^0C a	6.4	7.1	11.7	12.9	13.7	13.9	15.7	16.9	20	20.9	19.8	19	-	-
35^0C a	5	5.0	5.2	5.1	5.0	5.0	5	4.9	4.9	4.8	4.8	4.9	-	-

+++:> 1.5 x 10^5spores/ml, ++: 1.5 x 10^5 – 1 x 10^5spores/ml, +: < 1 x 10^5 spores/ml, - Nil. Means in a column followed by the common letters are not significantly different, p=0.05, LSD test

Results on the effect of pH on growth and sporulation of isolates of *C. beticola* presented in Table 4 showed that the best growth and good sporulation occurred at pH 6 (isolate A: 75 mm, isolate B: 74 mm)followed by pH 7 (isolate A: 70.6 mm, isolate B: 63.5 mm) suggesting that this pH range is suitable

for disease development, and cercosporin production which is pathogenic to the host.Poor growth with fewer spores was observed at pH 5 (isolate A: 61.5 mm, isolate B: 60.8 mm)and very little growth with no sporulation at pH 4 and pH 8. In pH3 and pH 9 there were complete inhibition leading to the death of the fungus. This suggests that too acidic and alkaline conditions retard the growth of *C. beticola*. The growth decreased as the pH moved away from 5.0 on either side. This indicated *C.beticola* has a very narrow pH range for growth and sporulation and thrives best under slightly acidic to neutral pH.The isolates of *C. beticola* were not significantly different in the growth at different pH compared to each other but the growth altogether differed at different pH.

Table 4: **Growth and sporulation of two isolates (A &B) of *Cercospora beticola* on spinach decoction agar at different pH, during 30 days of incubation at 25^{0}C**

pH	Average Colony mean diameter in mm at 5 days interval												Sporulation	
	5 days		10 days		15 days		20 days		25 days		30 days			
	A	B	A	B	A	B	A	B	A	B	A	B	A	B
3a	7.7	7.3	12.8	12.3	17.7	16.9	24.8	24	28.7	28.1	28.7	28	-	-
4bce	8.2	8	18.7	18.2	36.7	35.3	50.8	50.3	51.6	51.3	51.6	50.3	-	-
5bde	10.7	10.8	28.7	27.8	46.3	46.4	56	57.8	61.5	60.8	61.5	60.8	+	+
6bdf	12.5	12.8	38.3	38.6	55.4	54.9	70.9	72.8	75.2	74.3	75	74	++	++
7bdf	11.9	11.5	37.3	36.6	51.9	51.2	68.7	58.9	70.6	63.5	70.6	63.5	++	++
8bde	19.6	9.8	26.8	27.5	44.8	44.6	55.1	51.3	57.9	52.3	57.3	52.3	-	-
9 ac	7.8	7.4	14.6	14.8	21.3	20.9	30.2	35.7	32.4	37.5	32	37.4	-	-

+++:> 1.5 x 10^{5}spores/ml, ++: 1.5 x 10^5 – 1 x 10^{5}spores/ml, +: < 1 x 10^5 spores/ml, - Nil. In a column media followed by the same letters are not significantly (P = 0.05) different from each other according to Least Significant test.

Table 5: Growth and sporulation of two isolates (A &B) of *Cercospora beticola* on spinach decoction agar at different light regime during period of 30 days of incubation at 25^{0}C

Light regime	Average Colony mean diameter in mm at 5 days interval												Sporulation	
	5 days		10 days		15 days		20 days		25 days		30 days			
	A	B	A	B	A	B	A	B	A	B	A	B	A	B
Light **a**	13.3	13.6	34.8	40.8	57	57.9	65	68.5	78	79.5	78	79.5	++	++
Darkness **b**	12.2	12.6	22.8	27.8	32.4	33.8	45.4	43.8	52.5	59.3	52	59	-	-

+++:> 1.5 x 10^{5}spores/ml, ++: 1.5 x 10^5 – 1 x 10^{5}spores/ml, +: < 1 x 10^5 spores/ml, - Nil. In a column media followed by the same letters are not significantly (P = 0.05) different from each other according to Least Significant test.

The results on the effect of light regime on growth and sporulation of *C. beticola* presented in table 5 showed that the best mycelia growth was observed in culture kept in continuous light (isolate A: 78 mm, isolate B: 79.5 mm) while darkness (isolate A: 52 mm, isolate B: 59 mm) seems to inhibit the growth of the pathogen, and the growth at these two different condition was significantly different.*C. beticola*s populated well in light condition. Griffin (1994) stated that light stimulates reproduction of fungi more than growth. This stimulation howeverdepends on the nature of the medium on which the fungus is grown (Carlie, 1965).

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