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Short Communication

Effects of culture medium and lighting condition on induction of conidiation in *Cercospora citrullina*

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Abstract

Cercospora citrullina, isolated from pumpkin plant (*Cucurbita maxima*), initially could not produce conidia when cultured on potato dextrose agar, but was able to produce conidia when cultured on oatmeal agar, oat grain agar, wheat grain agar and barley grain agar. *C. citrullina* also produced conidia when cultured in substrates such as oat grain, wheat grain, barley grain and *Trichosanthes* sp. dried leaves. *Trichosanthes* sp. dried leaves induced the highest conidia production (4.81×10^3 conidia / ml) while oatmeal agar and barley grain agar yielded the best result among the agar media by inducing 1.23×10^3 conidia / ml each. Overall, continuous darkness condition was better than other lighting conditions (12 h near UV black light plus 12 h darkness condition; and 12 h fluorescent white light plus 12 h darkness lighting condition). Lastly, inoculation of *C. citrullina* onto cucurbit plants was successfully conducted by sandwiching inoculum between leaves and plastic cards.

Keywords: *Cercospora*, conidia, medium, lighting conditions, inoculation

1. Introduction

Cercospora citrullina Cooke causes the disease known as Cercospora leaf spot (CLS) among plants from the family Cucurbitaceae, such as watermelon, cucumber and squash (Chupp, 2006). The disease targets the foliage of the plant by forming circular spots with darkly pigmented margin together with light coloured centre (Kehinde, 2013). This will eventually lead to the defoliation of the host plant, resulting in a decrease in yield (Nelson, 2008).

C. citrullina is known to produce acicular, hyaline and multi-septated conidia arising from conidiogenous cells proliferating in sympodial style. The base of the conidia has darkly pigmented hilum (Hong, Park, Cho, & Shin, 2014; Mukhtar, Mushtaq, Khokhar, & Hannan, 2013; To-Anun, Hidayat, & Meeboon, 2011). Production of conidia is often observed on the host plant, whereas some *Cercospora* species only demonstrate vegetative growth on standard commercial medium. *C. citrullina* was found to be unable to produce conidia in artificial medium despite adjustment in parameters such as nutrients, temperature and lighting conditions (Goode & Brown 1970).

In this study, the best condition for conidia production of *C. citrullina* (isolated from leaves of pumpkin with CLS disease) was determined from among different culture media and substrates under varying lighting conditions.

2. Materials and Methods

2.1 Isolation of *Cercospora citrullina*

Cercospora citrulllina (Acc. no: KY 593165) was isolated from pumpkin leaves with CLS disease symptoms on 17th February 2015. The pumpkin leaves with CLS were collected from Kepala Batas, Penang, Malaysia. Single spore isolation was carried out to isolate the pure culture of *C. citrullina*. The isolated pure culture of *C. citrullina* was maintained in potato dextrose agar (PDA) (Oxoid PDA M0139, Basingstoke Hants, UK).

2.2 Greenhouse inoculation of *C. citrullina* to the cucurbit plants

The experiment was carried out using pumpkin,

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bitter gourd (*Momordica charantia* subsp. *charantia*), angled luffa (*Luffa acutangula* L. Roxb.), cucumber (*Cucumis sativus* L.) and watermelon (*Citrullus lanatus* (Thunb.) Matsum. & Nakai) (Soon Huat Seeds Co. Sdn. Bhd.). A piece of agar block (approximately 5 × 3 mm) was cut out from the 7-day old cultures of *C. citrullina* on PDA. The agar block was then transferred on a piece of sterile plastic card, 25 × 25 mm. The piece of agar block on the plastic card was sandwiched at the abaxial surface of the leaf and then fixed using a paper clip. Inoculations were made on 5 healthy leaves on 2 pumpkin plants per treatment. Control was prepared by fixing the plastic card using the paper clip with a piece of un-inoculated agar block on 5 healthy leaves on a pumpkin plant. A hundred microlitres of sterile distilled water was pipetted into the space between the plastic card and the abaxial surface of the leaf for both treatments. Inoculated plants and the control plant were covered in a plastic bag for 2 days. The same procedure was done for all cucurbits. The experiment was repeated twice for all samples.

2.3 Preparation of media and substrates

2.3.1 Preparation of *Trichosanthes sp.* leaves substrate

Trichosanthes sp. (Cucurbitaceae) leaves were collected in Universiti Sains Malaysia. The leaves were soaked in tap water overnight before cutting the leaves into small pieces approximately 10 × 10 mm. The cut leaves were air dried for about 6 to 8 hours under room temperature before being transferred into a 50°C incubator for 2 hours. The leaves were then taken out from the incubator and air dried overnight to ensure that the leaves were dried completely.

Three hundred milligrams of *Trichosanthes* sp. dried leaves was measured and put into a universal bottle. Likewise, 0.6 ml of distilled water was added into the universal bottle containing *Trichosanthes* sp. dried leaves. *Trichosanthes* sp. dried leaves were sterilized by autoclaving at 121 °C for 20 minutes.

2.4 Preparation of oat grain, wheat grain and barley grain substrates

Three grams of oat grains was measured and put into a universal bottle. Six millilitres of distilled water was added into the universal bottle containing the oat grain. The oat grains were sterilized by autoclaving at 121 °C for 20 minutes. Wheat grain and barley grain substrates were prepared using the same method.

2.5 Preparation of oatmeal agar (OA)

Seventy-two grams of oatmeal agar (BD Difco™ Oatmeal Agar, New Jersey, USA) was measured and suspended in 1000 ml distilled water. The mixture was heated and stirred until the agar was evenly distributed in the distilled water and then autoclaved at 121 °C for 20 minutes.

2.6 Preparation of cornmeal agar (CMA)

Seventeen grams of cornmeal agar powder (Oxoid CM103, Basingstoke Hants, UK) was suspended in 1000 ml of distilled water. The medium was heated and stirred to dissolve the powder completely. Then, the medium was autoclaved as in the above-mentioned condition.

2.7 Preparation of oat gain agar (OGA), wheat grain agar (WGA) and barley grain agar (BGA)

Thirty grams of oat grains was weighed and added into a flask. Half a litre of distilled water was added into the flask. The flask was sealed and boiled at 100 °C for 45 minutes. The oat grains were filtered out and discarded. The filtered broth was transferred to a flask and distilled water was added to the flask until 1000 ml of broth was obtained. Eighteen grams of agar (Oxoid LP0011, Basingstoke Hants, UK) was added into the flask containing the 1000 ml of broth then autoclaved as mentioned above condition. The same method was used to prepare wheat grain and barley grain agar.

2.8 Inoculation of *C. citrullina* onto various types of agar media and substrates and incubation under different lighting condition.

2.8.1 Lighting condition treatments

Cercospora citrullina was inoculated onto each type of media and substrates and incubated under three different lighting conditions: 12 h near UV black light (F20T9/BL 18 W, Hitachi, Ltd, Tokyo, Japan) (Peak wavelength: 352 nm) irradiation plus 12 h of darkness (LD 12:12); 12 h fluorescent white light plus 12 h darkness (FWL 12:12) (Master TL-D Super 80 18 W/865, Philips Lighting, Amsterdam, Netherlands) (Peak wavelength: 450 nm); and complete darkness for 14 days. Three replicates were prepared for each type of media and substrates and for each lighting condition. The experiment was repeated twice.

LD 12:12 lighting condition was achieved by placing the inoculated substrates or media inside a chamber with black light irradiation for 12 h and darkness for another 12 h. FWL 12:12 lighting condition was set by placing the cultures in a chamber with fluorescent white light irradiation for 12 h and darkness for another 12 h. Lastly, complete darkness condition was achieved by putting the cultures into an opaque, tightly locked storage box.

2.9 Inoculation of *C. citrullina* onto media

A piece of agar block, approximately 5 × 3 mm in diameter, was cut from a 7-day old PDA culture and transferred into oatmeal agar. The plate was then sealed with parafilm (Parafilm M™ Wrapping Film, Thermo Fisher Scientific, MA, USA). CMA, OGA, WGA and BGA were inoculated with *C. citrullina* using the above-mentioned method.

2.10 Inoculation of *C. citrulllina* onto substrates

10^5 hyphal fragment / ml of *C. citrullina* hyphae suspension was made by flooding 7-day old *C. citrullina* PDA plates with 10 ml sterile 0.01% Tween™ 20 *(*ACROS Organics™, Thermo Scientific, MA, USA). The concentration of the hyphal suspension was measured using heamacytometer. A hundred microlitres of hyphal suspension was pipetted into each bottle of *Trichosanthes* sp. dried leaves.

After adding the hyphal suspension, each bottle was shaken for 10 seconds to ensure the hyphal suspension was spread evenly onto the leaves. The bottle was then sealed with parafilm. The same inoculation method was applied to oat grain, wheat grain and barley grain substrates respectively.

2.11 Assessment of conidia production of *C. citrullina*

2.11.1 Assessment of conidia production of *C. citrullina* on media

The *C. citrullina* colony on the agar plate was flooded with 10 ml of 0.01% sterile Tween™ 20 and then scratched by using an inoculation wire loop. A hundred microlitres of the suspension was withdrawn using a micro-pipette and pipetted into a haemacytometer (Neubauer-improved haemacytometer, Marienfeld, Lauda-Königshofen Germany). The concentration of conidia produced was measured using a haemacytometer.

2.11.2 Assessment of conidia production of *C. citrullina* on substrates

Ten millilitres of 0.01 % sterile Tween™ 20 was poured into the bottle containing culture of *C. citrullina* in substrate. The suspension was mixed by using an inoculation wire loop. A hundred microlitres of the suspension was withdrawn using a micropipette and pipetted into a haemacytometer. The concentration of conidia produced was measured using a haemacytometer.

2.12 Statistical analysis

Kruskal- Wallis H test was used to analyse the concentration of conidia produced on various type of media and substrates under different lighting conditions. The test was conducted using SPSS version 22 (IBM Corp. Released 2013. Armonk, NY). The significant level used for the analysis was $\alpha = 0.05$.

3. Results

3.1 Greenhouse inoculation of *C. citrullina* to the cucurbit plants

Disease symptoms started to appear as soon as 2 days after inoculation for all plants, though lesion-distinct dark edges and discolouration were yet to be seen. After 10 days of inoculation, lesions with prominent CLS disease symptoms were developed. The disease lesions observed from the pumpkin, bitter gourd, cucumber, angled luffa and watermelon leaves were found to be very much similar. The discolouration was observed at the edge of the disease lesions beside brown to greyish necrotic regions in the middle of the lesions (Figure 1). Controls in neither repetition resulted in any CLS disease symptoms.

3.2 Assessment of conidia production of *C. citrullina*

The *C. citrullina* isolate was not able to produce conidia on PDA. White cottony colony was formed when the isolate was cultured on PDA, without conidia production. Likewise, no conidia production was found when the isolate was cultured on another commercial medium, which is the CMA. The only commercial medium which induced conidia production in *C. citrullina* was OA. Reddish-orange pigmentation was found at the reverse of *C. citrullina* colony cultured on OA (Figure 2). Apart from the commercially available OA, homemade media including OGA, WGA and BGA were also able to induce production of conidia in *C. citrullina*. The reverse of colonies was pigmented when *C. citrullina* was cultured on OGA, WGA and BGA (Figure 2).

On the other hand, all the substrates used in this study, namely oat grain, wheat grain, barley grain and *Trichosanthes* sp. dried leaves induced conidia production in *C. citrullina* under all lighting conditions.

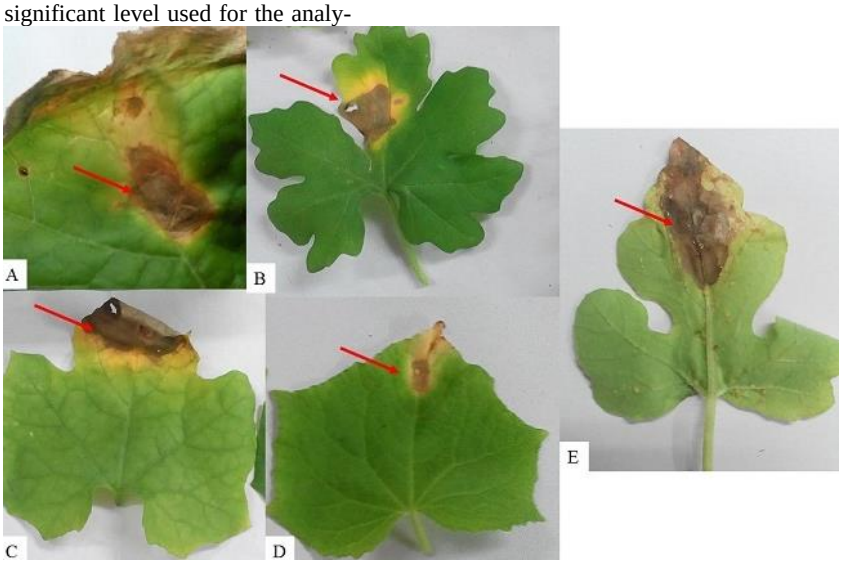

Figure 1. CLS disease lesion formed by the inoculation of *C. citrullina* (Red arrow). A: Pumpkin leaf. B: Bitter gourd leaf. C: Angled luffa. D: Cucumber E: Watermelon.

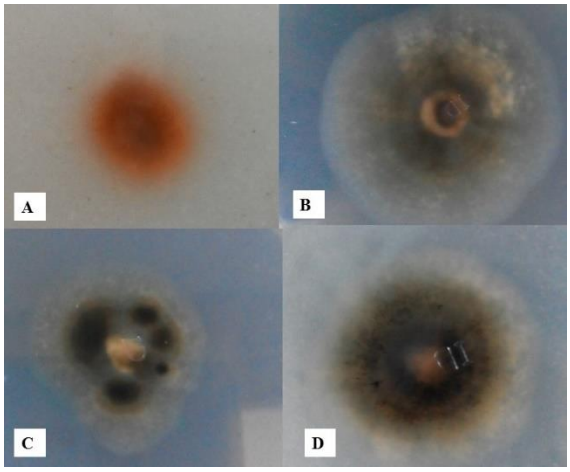

Figure 2. The reverse of *C. citrullina* colony cultured on various type of media**.** A: Oatmeal agar B: Oat grain agar C: Wheat grain agar D: Barley grain agar**.**

The conidia of *C. citrullina* produced on the agar and substrates exhibited similar morphology. The conidia were smooth, acicular and multi-septated. In addition, distinct darkly pigmented hila were found on the conidia produced by *C. citrullina* cultured on the agar and substrates (Figure 3).

3.3 Statistical analysis

Statistical analysis by Kruskal-Wallis has shown that there was no statistical difference in the concentration of conidia induced by the media and substrates used in the experiment under LD 12:12 irradiation, $\chi^2(8) = 12.067$, $p = 0.148$ (Table 1). On the other hand, significant differences were identified in the concentration of conidia induced by the media and substrates used in the experiment under FWL 12:12, $\chi^2(8) = 19.903$, $p = 0.011$ and continuous darkness condition, $\chi^2(8) = 22.223$, $p = 0.005$. WGA, BGA and barley grain induced the highest conidia production under FWL 12:12 condition with a mean of 0.62×10^3, 1.23×10^3 and 1.36×10^3 conidia / ml respectively. OA, OGA, oat grain, wheat grain and *Trichosanthes* sp. dried leaves gave the highest conidia concentration under the continuous dark condition which are 1.23×10^3, 0.740×10^3, 3.33×10^3, 1.36×10^3 and 4.81×10^3 conidia / ml respectively. Overall results showed that *Trichosanthes* sp. dried leaves were most effective in terms of conidia induction (4.81×10^3 conidia / ml). Among the agar media, commercial medium, OA and homemade medium, BGA induced higher conidia production, with a mean of 1.23×10^3 conidia / ml.

4. Discussion

Environmental factors such as temperature, relative humidity, availability of nutrients as well as lighting condition influence the sporulation, conidial germination and growth of fungi (Dahlberg & Etten, 1982; McQuilken, Budge, & Whipps, 1997). In our study, the focus was directed to the influences of culture media and the lighting condition in the conidiation of *C. citrullina*. Beckman and Payne (1982) showed that spore production of *Cercospora zeae-maydis* Tehon & E.Y. Daniels was influenced by both the culture medium and lighting condition. In their study, green-corn decoction agar amended with $CaCO_3$ produced a higher amount of spore than a medium prepared using the V-8 juice under 11 days of constant fluorescent light followed by 3 days of darkness condition. Apart from that, some of *Cercospora* spp. were reported to be unable to produce conidia when cultured on standard laboratory media and efforts have been made to induce conidiation in these organisms. For example, *Cercospora kikuchii* (T. Matsumoto & Tomoy), which only demonstrated vegetative growth on standard culture media, was tested with several media prepared from immature and senescent tissues of alfalfa corn, cotton and wheat as well as

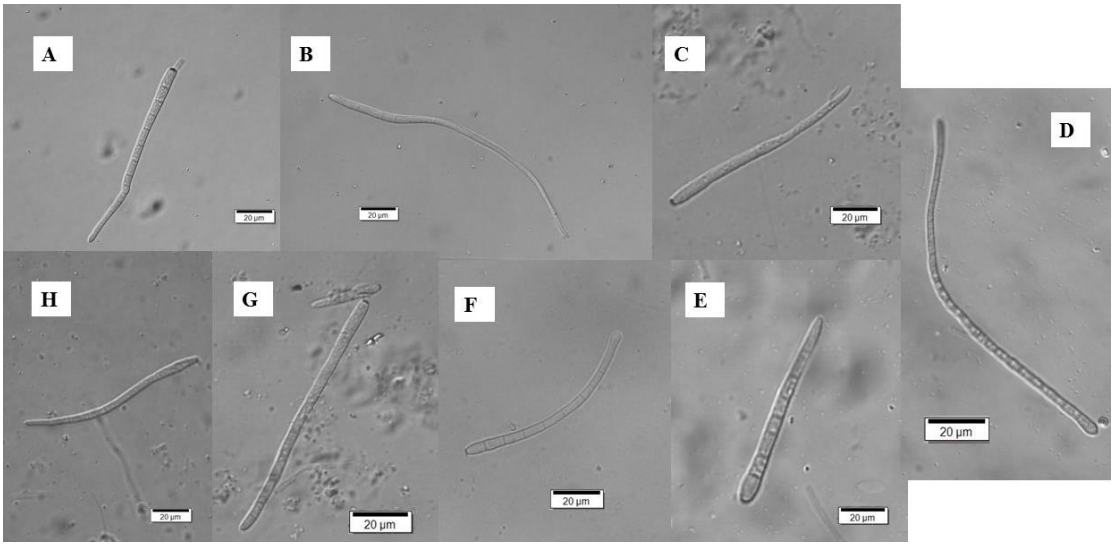

Figure 3. Conidia of *C. citrullina* produced on various type of media and substrates. A: Oatmeal agar (OA) B: Oat grain agar (OGA) C: Wheat grain agar (WGA) D: *Trichosanthes* sp. dried leaves E: Oat grain substrate F: Barley grain substrate G: Barley grain agar (BGA) H: Wheat grain substrate.

Table 1. Effects of different media and substrates on *C. citrulline* conidiation under different lighting conditions.

Medium	Concentration of conidia								
	LD 12:12			FWL 12:12			Dark		
	Mean ± SD[a]	Mean rank[b]	Comparison of mean rank[c]	Mean ± SD[a]	Mean rank[b]	Comparison of mean rank[c]	Mean ± SD[a]	Mean rank[b]	Comparison of mean rank[c]
Oatmeal (OA)	0.37 ± 0.37	14.83	-	0.37 ± 0.37	7.67	abc	1.23 ± 0.21*	15.67	bcde
Oat grain agar (OGA)	0.00 ± 0.00	6.00	-	0.25 ± 0.04	6.33	ab	0.74 ± 0.37*	9.83	abc
Wheat grain agar (WGA)	0.25 ± 0.04	11.67	-	0.62 ± 0.21*	10.50	abcd	0.37 ± 0.37	6.00	ab
Barley grain agar (BGA)	0.37 ± 0.00	15.50	-	1.23 ± 0.21*	19.67	cd	1.11 ± 0.37	14.17	abcde
Cornmeal agar (CMA)	0.00 ± 0.00	6.00	-	0.00 ± 0.00	3.50	a	0.00 ± 0.00	2.50	a
Oat grain	0.99 ± 0.77	21.83	-	1.60 ± 0.57	22.50	d	3.33 ± 0.37*	23.33	de
Wheat grain	0.37 ± 0.37	14.83	-	1.11 ± 0.37	17.67	bcd	1.36 ± 0.21*	17.33	bcde
Barley grain	0.49 ± 0.21	18.00	-	1.36 ± 0.21*	21.33	d	0.86 ± 0.57	11.50	abcd
Trichosanthes sp. dried leaves	0.49 ± 0.43	17.11	-	1.23 ± 0.85	16.83	bcd	4.81 ± 1.93*	25.67	e

[a]Mean concentration of conidia ($\times 10^3$) (conidia / ml) (n = 3)
[b]Mean Rank from Kruskal-Wallis test ($\alpha = 0.05$).
[C] Mean rank was compared by using pairwise comparison. Differences significant at $\alpha = 0.05$. Same alphabet indicates no statistical significant difference while different alphabet was assigned when statistical significant difference was found.
*Highest concentration of conidia produced among three lighting conditions for that medium or substrate.

agar made by decoctions of carrot leaves (Vathakos & Walters, 1979). This observation was corroborated by findings in our study, in which *C. citrullina* was not able to produce conidia when cultured on PDA. However, induction of conidia was observed for other media and substrates prepared by using oat grain, wheat grain and barley grain, as well as *Trichosanthes* sp. dried leaves or commercially available oatmeal agar. The genus *Trichosanthes*, belonging to the family Cucurbitaceae which also serves as a hosts of *C. citrullina*, was found to be the most effective culture medium for the induction of conidia in *C. citrullina*. In fact, the use of host plant as the medium for conidia induction in plant pathogenic fungi is common (Su, Qi, & Cai, 2012). This suggested that the incorporation of the host plant in the culture can be an ideal alternative to induce conidiation in *Cercospora* spp. which do not produce conidia under common medium such as PDA. Apart from this, our study found that agar and substrates prepared by using cereal grains such as oat grain, wheat grain and barley grain were effective in *C. citrullina* conidia induction. This result corresponds to the finding of Djébali, Gaamour, Badri, and Aouani (2010), in which solid wheat bran juice was able to induce sporulation in 2 out of 4 strains of *Cercospora medicaginis* Ellis & Everh. Sorghum was found to be highly effective in the induction of conidia production in *Cercospora canescens* Ellis & G. Martin, which causes CLS disease in mungbean crops (Chand, Kumar, Singh, & Pal, 2013). Therefore, incorporation of cereal grains in the preparation of medium is useful for conidia induction in *Cercospora* spp.

Light irradiation is often deemed crucial in the conidiation of many *Cercospora* spp. For instance, 5 isolates of *C. kikuchii* produced conidia under 12 h of light and dark alternating lighting condition but conidia were scarcely found under continuous darkness condition (Yeh & Sinclair, 1980). Although some studies found that lighting condition is one of the major contributing factors towards the conidiation in many *Cercospora* spp., the study by Abdou and Cooper (1974) demonstrated a different scenario. In their study, *Cercospora arachidicola* Hori did not require specific lighting condition for conidiation but light exposure is a must for conidiation in *Cercospora personatum* (Beck & Curtis). Besides this, conidiation of *C. kikuchii* was found strongly suppressed under continuous lighting conditions (Bluhm, Burnham, & Dunkle, 2010). Instead, the formation of melanised hyphae which was regulated by the circadian rhythm was observed after 4 days of 12 h of light and 12 h of dark incubation. In contrast, pigmentation was only observed on the reverse of *C. citrullina* colony cultured on OA, OGA, WGA and BGA and the colouration was exhibited regardless of the type of lighting conditions in our study. In addition, the reddish-orange colouration found at the reverse of *C. citrullina* colony cultured on the OA was very much similar to the red pigmentation found on *Cercospora nicotianae* Ellis & Everh. culture. Thus, the pigmentation might be associated with the production of photoactivated toxin, cercosporin (Daub & Chung, 2007).

Lastly, the time required for the appearance of disease symptoms was shortened by using the inoculation method mentioned in this study as the formation of disease lesion was found within 10 days. Likewise, retention of high humidity was achieved using this inoculation method. The study by Kumar, Pandey, and Chandra (2011) proved that relative humidity (RH) ranging from 92 to 100 % supported the initiation of germtube of *Cercospora canescens* Ellis & G. Martin conidia at 5 to 35 °C. Their findings suggested that RH plays an important role in the pathogenesis of *Cercospora* spp.

Covering the inoculated plants with plastic bags alone might not be enough to maintain such high humidity condition. Placing the agar blocks containing the inoculum between the abaxial surface of the leaf and a piece of plastic card managed to prevent the evaporation of water thus created a moist-chamber-like condition. This condition favours the penetration of the fungal pathogen as desiccation will lead to the death of the pathogen (Agrios, 2005).

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