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Effect of culture media, temperature, light and pH on *Corynespora cassiicola*; A fungal pathogen of leaf spot disease on *Carica papaya*.

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There are many factors influencing the ability of fungal pathogen pathogenicity. It is critical to understand the characters and the behavior of the pathogen on environmental factors influences in order to design suitable control methods for specific pathogen. In this study, different media, temperature, light density and pH levels were tested for their influence toward the *Corynespora cassiicola,* the causal agent for leaf spot disease of papaya. Six different media were tested in this study. Based on the result, the most suitable media for the *C. cassiicola* growth were Potato Dextrose Agar (PDA) half Strength and NA with 31.83 and 31.46 AUGC respectively. Meanwhile, Corn Meal Agar (CMA) showed the worst performance among those media tested with 24.09 AUGC value. Temperature of 30°C is the best for *C. cassiicola* growth and 12h UV 12h Light was the best combination for *C. cassiicola* spore production. Other than that, pH range level from 7 to 8 shows the best pH level for both growth and sporulation of *C. cassiicola*.

Keywords: **Leaf Spot Disease, *Corynespora cassiicola*, Plant Disease, *Carica papaya*, Temperature, pH, Light, Leaf Spot Disease, Papaya. Plant Pathology.**

INTRODUCTION

There are many factors influencing the dissemination of disease cause by biotic agent such as *C. cassiicola*. It is critical to understand the characters and the behavior of the pathogen to make the best strategies precaution and prevention in order to avoid mass crops destruction.

Good understanding of the pathogen character and the related factors that help the disease to well-developed could save a million of ringgit in yield losses. It is not only reflects farmers, in some cases the diseases development can also cause damage to the environment's ecosystem. The worst-case scenario would be the uncontrollable diseases in crops which cause starvation. As the *C. cassiicola* was the new re-emergence fungi causing the leaf spot disease, it is critical to explore the factor contributing to the causal of fungal infection in papaya. This study

presented the cause of the fungi that could be destructed in order to minimize the effects of the infection.

As for example there are many different level of optimum temperature for *C. cassiicola* was reported. (Ahmed et al., 2013; Fernando et al., 2012; Madhavi and Murthy, 2001 report was at 30°C. The same thing goes to pH and and media. For that reason, basic factors such as culture media, temperature, light and pH is important to be test in this study.

Therefore, the objective of this study is to identify the environmental factors which influence the *C. cassiicola,* a pathogen of leaf spot disease of *C. papaya*.

MATERIALS AND METHODS

Effect of Media on *C. cassiicola* Growth, Spore Production and Spore Germination

C. cassiicola Growth

Potato Dextrose Agar (PDA), Potato Dextrose Agar Half Strength (PDA-HS), V-8 Juice (V8A), Corn Meal Agar (CMA), Nutrien Agar (NA), Oat Meal Agar (OMA) and Sabaroth Agar (SA) were used in this experiment as media for the growth of *C. cassiicola*. All the media were prepared as per manual.

C. cassiicola Sporulation

A 5 mm agar plug from a 7-day old culture was placed in the center of plates of PDA, NA, PSA (PDA + Sukrose), V8 Juice Agar, and CMA. The plates were sealed with parafilm and incubated at 30°C under NUV light, 12h light and 12 h Dark (Hailmi et al., 2011; Ng et al., 2011).

The light duration was controlled by a 24 h regimen time controller (Theben-Werk Zeitantomatik Gmoh, Hohenbergstrabe 32, D72401 Heigerloch, and Germany). Conidia production was assessed 15 days after media inoculation. The conidia were harvested by flooding the medium with 15 mL sterile water and scraping the conidia off the agar surface with rubber spatula, followed by another 5 mL sterile water to flush out any residual conidia from the medium. The conidia were counted using a Brite-line phase contrast hemacytometer.

Effect of Temperature on the Growth, Spore Production and Spore Germination of *C. cassiicola*

C. cassiicola Growth

Five mm plug was taken from the margin of an actively growing culture (10 days old) and inoculated on PDA. The plug was placed in the centre of each petri plate then the plates were sealed with parafilm and incubated in the incubator at various temperatures (20°C, 25°C, 30°C and 35°C). The radial growth of the isolates were measured daily for eleventh days using a pair of callipers.

C. cassiicola's Conidia Germination

A 1 mL spore suspension containing +- 10^4 conidia was spread on the surface of water agar (WA) medium in a petri plate with 4 replications in a completely randomized design (CRD). The petri plates were sealed with parafilm and incubated for 24 h at difference temperatures; 20°C, 25°C, 30°C and 35°C. The percentage germination of the spores was calculated based on random samples of 200 spores.

C. cassiicola's Sporulation

A 5 mm agar plug from a 7-day old culture was placed in the center of plates of PDA. The plates were sealed with parafilm and incubated at 30°C. Conidia production was assessed 15 days after media inoculation harvested using rubber spatula as previous methods.

Effect of Light on *C. cassiicola* Sporulation

C. cassiicola Growth

Five different light intensities were exposed to the *C. cassiicola***.** The intensities of light selected were 12 hour light and 12 hours dark (12HL12HD), 24 hours light (24HL), 24 hours dark (24HD), were tested in this experiment. The growth of *C. cassiicola* was taken daily.

C. cassiicola's Sporulation

The light intensity was as mention in previous experiment with additional of 12 hours ultra violet and 12 hours dark (12HUV, 12HD), and 12 hours ultra violet and 12 hours light (12h UV, 12h L). The *C. cassiicola* was then harvested by rubbing the surface of *C. cassiicola* colony using scrpping spatula with 10 ml of sterile distilled water for petri disk. Harvested conidia were then counted using hemacytometer.

C. cassiicola's Conidia Germination

The light intensities was as mention in previous experiment with additional of 12 hours ultra violet and 12 hours dark (12h UV, 12h D), and 12 hours ultra violet and 12 hours light (12h UV, 12h L). The *C. cassiicola* was then harvested by scrapping the surface of C*. cassiicola* colony using scrpping spatula with 10 ml of sterile distilled water for petri dish. Harvested conidia were then counted using hemacytometer.

Effect of PH on *C. cassiicola* Growth, Spore production and Spore Germinations

Effect of pH on C. cassiicola Growth

Difco's Potato Dextrose Agar (PDA) was prepared for 100 mL for each pH. The pH was adjusted accordingly by using HCL and NaOH. Then the solution was autoclaved and poured into petri dishes, a 0.5cm fungal disc was taken from the periphery of 7-day-culture and transferred to the center of agar. pH 4, pH 5, pH 6, pH 7 and pH 8 were tested to determine the optimum pH condition for fungal growth. Then the petri dishes were incubated at 30°C with four replicates for each temperature. Radial growth was measure daily using ruler for 7 days.

Effect of pH on C. cassiicola's conidia germination

Water agar (WA) was used instead of PDA because WA is non-nutritious. Thus the fungal growth after conidia germination is slower and the counting of the *C. cassiicola* germinating conidia can be performed more easily. Conidia germination was observed using a compound microscope at 40x magnification.

The water agar was adjusted to 5 different levels of pH in the range of 5.0-9.0. Source of conidia was obtained from previous experiment. Conidia suspension in H_2O were pooled and stirred evenly, then, about 0.5ml of the conidia suspension was spread onto the treated water agar using 'L' shape (hockeystick) glass rod. The experiment was replicated 4 times with twice repetitions. Treatments were arranged in a completely randomize design (CRD) and incubated in a room temperature for 24h. Conidia germination was assessed after 24h incubation. Conidia were considered germinated when the germination tubes were half the length of the conidia.

RESULTS

Effect of Media on *C. cassiicola* Growth, Spore Production anda Spore Germination

Six agar media were tested in this experiment to study fungi growth stability in the laboratory.. Based on the result, the most suitable media for the *C. cassiicola* growth were PDA (half Strength) and NA (Table 1) with 31.83 and 31.46 AUGC respectively. (Ln value Y= 0.267+0.838x; R^2 = 0.995) for PDA-HS (Figure 1). The result (Table 1) shows the significant different of both media ($P<0.05$) compared to the rest media tested. It was followed by PDA, V8-Agar and SBA. The CMA showed worst performance among those media tested with 24.09 AUGC value (Table 1). Ln value for CMA was Y=7.777+0.792x; R^2=0.995.

Experiment on suitability of the media for *C. cassiicola* sporulation was also conducted. Clealy the result shows there is effect of different media tested on the sporulation ability of *C. cassiicola*. Result from this experiment shows PDA-HS has a best performance on *C. cassiicola* sporulation. With 128,125 spores harvested (Table 1) per plates and it was the highest number of spore with significant different ($P<0.05$) compared to PDA (Table 1) as a second best media for *C. cassicola* sporulation. Other medias produced much lower number of spores per plate as SBA SHOWED the lowest performance (Figure 1) with only 18,125 (Table 1) spored harvested.

Table 1: Area Under Growth Curve (AUGC) on the effect of different agar media on the Growth of *C. Cassiicola* incubated at 25°C.

Media	Growth AUGC (Cm^2)	Concentration (Spore/Ml)
NA	31.46^{a}	$51,250^{c}$
PDA	29.19^{ab}	$88,125^{b}$
PDA-HS	31.83^{a}	$128,125^{a}$
V8 Agar	24.15^{d}	$8,125^{d}$
SBA	25.40^{c}	$3,125^{d}$
CMA	24.09^{d}	$4,8750^{c}$

[x] **AUGC** : Area under growth curve

[z] : Different letters show the significant difference between treatments, value followed by same letter are not significantly different according to least significant difference test ($p<0.05$)

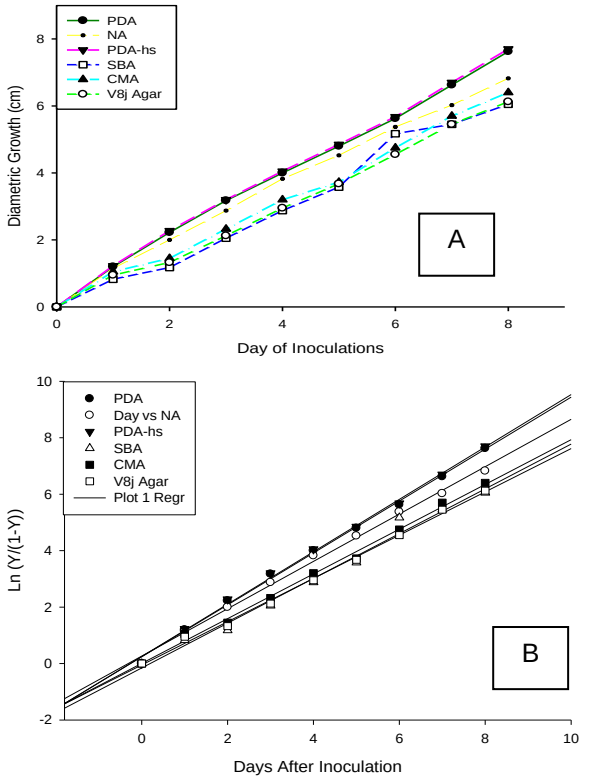


Figure 1: Radial growth of *C. cassiicola* on different culture media at 30°C: (A) untransformed values, (B) Regression of transformed data using the logistic model ln (Y/(1-Y)).

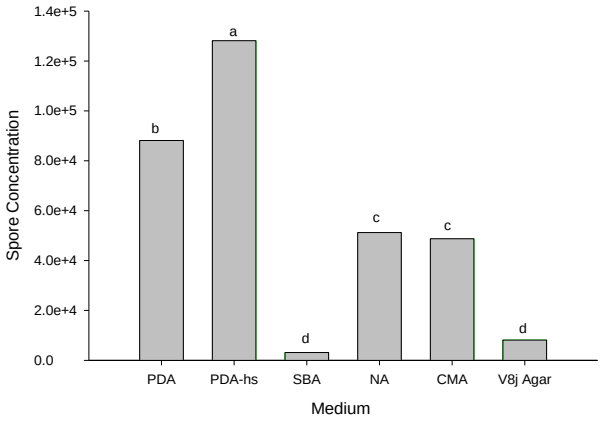


Figure 2: Effect of different culture media on *C. cassiicola* sporulation Incubated on 30°C.

Effect of Temperature on Growth, Spore Concentration and Spore Germination of *C. cassiicola*.

Temperature is one of the most important environmental factors that influences the growth, germination and pathogenicity of fungi. It was critical to understand the ability of *C. casiicola* as pathogen on different temperature in order to minimize the potential of fungi as pathogenic pathogen. It was clearly proved that the growth of *C. cassiicola* was highly influenced by the difference temperature (Table 1). Temperature ranged from 20°C to 30°C seems the best growth condition for *C. cassiicola* to grow with no significant effect (P>0.05) (Table 1). Temperature at 35°C shows the less growth of *C. cassiicola* with significant different (P<0.05) compared to other temperatures. The temperature of 40°C completely suppressed the growth of fungi.

Figure 2 shows the effects of temperature on *C. cassiicola* sporulation. Temperature affected the number of spore production that are 92,205 and 119,520 spore per plates under 25°C and 30°C, respectively which did not show significant effects. Significant different was observed at both 25°C and 30°C compared to 20°C and 35°C that showed with different alphabet (Table 1 and Figure 3).

Base on the result of this experiment, temperature clearly played an important role on the germination of *C. cassiicola*. Temperature at 25°C and 30°C showed the best growth temperature for to the germination of *C. cassiicola* conidia compare to other temperature levels (20°C and 35°C) with 81.00% and 79.75% respectively (Table 1, Figure 4). The lowerst germination of conidia was at 35°C with 49.75% and it was statistically different compared to 20°C which recorded 58.75% of conidia germination (Table 1, Figure 4).

Table 2:; Effect of difference temperature on the growth, spore production and spore germination of *C. cassiicola*. The fungi were incubated on PDA for the growth and spore production experiment and on WA for spore germination test.

Temperature	Growth AUGC (cm^2)	Concentration (Spores/ml)	Germination Mean (%)
20˚C	22.98^{ab}	6,403^{b}	58.75^{b}
25˚C	30.45^{a}	92,025^{a}	81.00^{a}
30˚C	28.85^{a}	119,520^{a}	79.75^{a}
35˚C	12.78^{c}	32,028^{b}	49.75^{c}
40˚C	-	-	-

AUGC : Area under growth curve

a,b,c : Different letters show the significant difference between treatments, value followed by same letter are not significantly different according to least significant difference test (p=0.05)

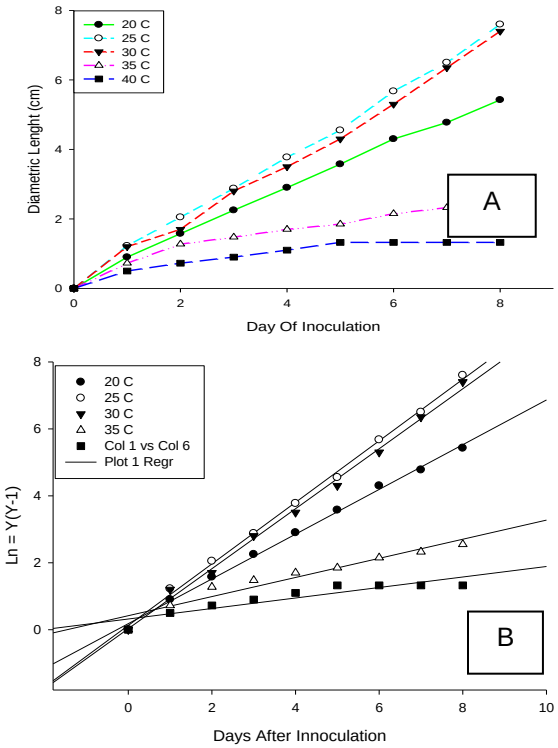


Figure 1: Radial growth of *C. cassiicola* on different Temperatures: (A) untransformed values, (B) Regression of transformed data using the logistic model ln (Y/(1-Y)).

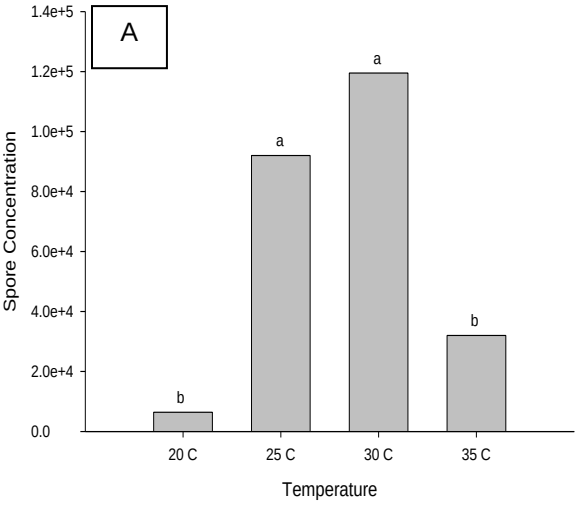


Figure 4: Effect of temperature on sporulation *of C. cassiicola* on PDA (HS) media.

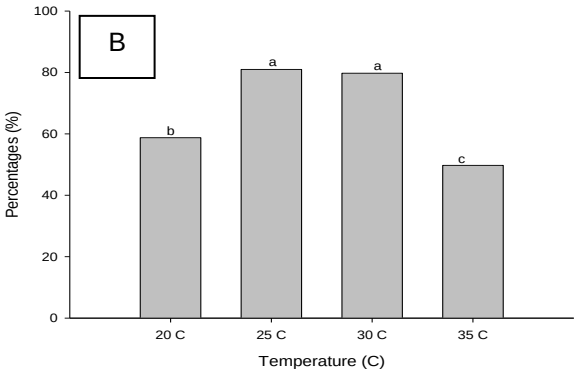


Figure 5: Effect of temperature on the germination of *C. cassiicola* on PDA-HS in incubator.

Effect of Light on the Radial Growth, Spore Production and Spore Germination of *C. cassiicola*.

Effect of five different intensities of light on growth *of C. cassiicola* was studied in this experiment. The 12HL12HD, 24HL, 24HD light treatments show the different on the Area under Growth Curve (AUGC) with 30.46, 31.46 and 30.38 respectively (Table 2, Figure 5). But these differences did not show significant different (P>0.05) s shows in figure 6.

Effect of different light intensities were tested on the ability of the production of spores of *C. cassiicola*. The result shows an exposer of the *C. cassiicola* to 12HUV12HL has proven as the best media for *C. cassiicola* to produce conidia. It was statictically significant (P<0.05) compared to other treatments (Table 2, Figure 6). It were followed by 12HUV12HD and 24HL that showed no significant between them The treatment of 24HD produced the lowest spore production at 16,250 spores per plates **(**Table 2, Figure 6).

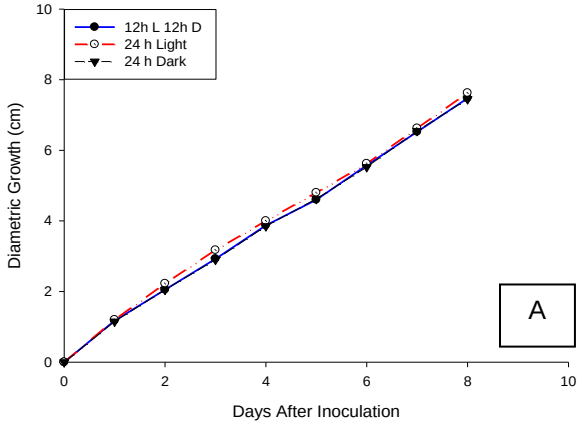
Difference intensities of light on the germination of *C. cassiicola* conidia were stated in Table 3. The experiment covers five difference lighting levels as the previous experiment. Intensities of light as shows in Table 2**,** did not influence the ability of the conidia to germinate and showed no significant different compared to other conditions (Figure 7).

Table 3: Influence of light on radial growth, spore production and spore germination of *C. cassiicola* that were incubated on PDA-HS and on WA.

Density of Light	Growth AUGC (cm^2)	Concentration (Spores/ml)	Spore Germination
12H Light 12H Dark	30.46[a]	48,125[c]	80.25[ab]
24H Light	31.46[a]	78,125[b]	73.5[c]
24H Dark	30.38[a]	16,250[d]	82.75[a]
12H UV 12H Dark	-	88,125[b]	83.25[a]
12H UV 12H Light	-	**110,625[a]**	75.75[bc]

AUGC : Area under growth curve

z : Different letters show the significant difference between treatments, value followed by same letter are not significantly different according to least significant difference test (p=0.05)



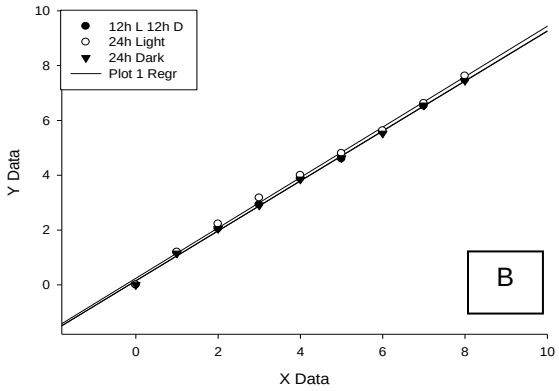


Figure 2: **Radial growth of *C. cassiicola* on different light densities at 25°C: (A) untransformed values, (B) Regression of transformed data using the logistic model ln (Y/(1-Y)).**

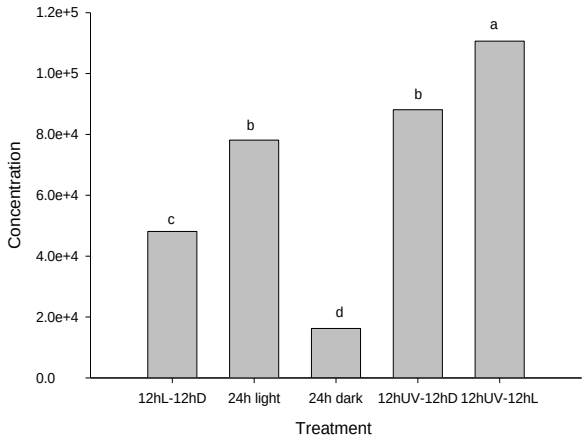


Figure 7: Effect of difference density of light exposure on the spore production of *C. cassiicola* incubated on PDA-HS. The significant difference was mark as different alphabet.

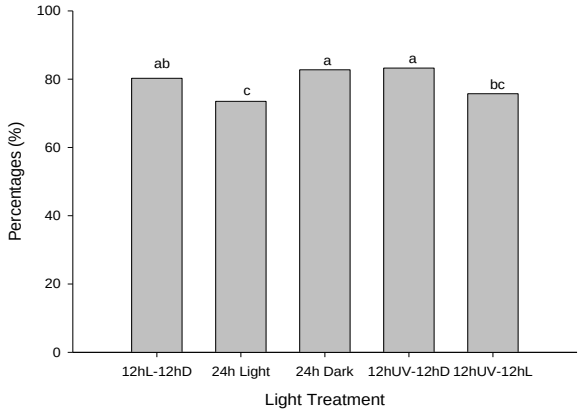


Figure 8: Effect of difference period of light exposure on the spore germination of *C. cassiicola* incubated under 25°C on Water Agar media.

Effect of pH on *C. cassiicola* growth, Sporulation and Spore Germination.

Influence of pH on *C. cassiicola* growth, sporulation and spore germination was tested in this study. Five level of pH which was pH5, pH6, pH7, pH8 and pH9 were selected for the study. As could be show on Table 4**,** pH7 to pH8 were proved to be the best pH for *C. cassiicola* growth. It was based on the value of AUGC with 31.18 and 30.73 respectively with significant different compared to others pH level tested (Table 4, Figure 9)**.** The lowest AUCG was recorded at pH5 with 20.96 follow by pH9 with 27.10 value of AUGC as second lowest.

Bar chart in Figure 10 was the result of influence

of pH on *C. cassiicola* sporulations. It was clearly show the differences among pH level treatment. pH 7 and pH 8 were shows a significant different (P<0.05) compared to other pH level treated. With 127,500 and 118,750 spore concentration per plate for pH7 and pH8 respectively, it was wide difference compare to spore production on pH6 and pH9 as statistically second best of spore production per plates (Table 4, Figure 10). Meanwhile the lowest production was on the pH5 with only 18,750 spore concentration per plate.

pH7 and pH8 consistently seem to be the best level of pH for the growth, spore production and also in this experiment which was testing the pH level on conidia germination (Table 3, Figure 11). pH7 and pH8 shows highest mean percentages with 81.25% and 79.25% respectively. It was followed by pH6 as second best level of pH for sporulation with 58.75% and pH5 and pH9 as the lowerts number of conidia germination with 43.50% and 49.75% respectively (Table 3, Figure 10).

Table 4: Influence of pH on *C. cassiicola* growth on different pH level on PDA-HS Media incubated at 25°C.

pH Level	Growth AUGC (cm2)	Concentration (Spores/ml)	Germination Mean (%)
pH 5	20.96[d]	18750c	43.50[c]
pH 6	28.29[b]	41875[b]	58.75[b]
pH 7	31.18[a]	127500[a]	**81.25[a]**
pH 8	30.73[a]	118750[a]	**79.25[a]**
pH 9	27.10[c]	47500[b]	49.75[c]

AUGC : Area under growth curve

[z] : Different letters show the significant difference between treatments, value followed by same letter are not significantly different according to least significant difference test (p=0.05)

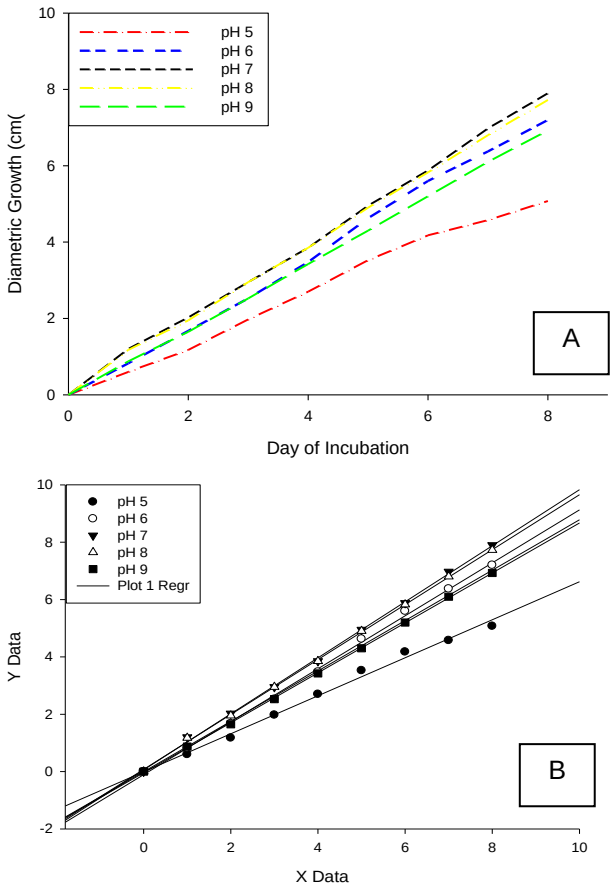


Figure 9: Radial growth of *C. cassiicola* on different pH at 30°C: (A) untransformed values, (B) Regression of transformed data using the logistic model Ln (Y/(1-Y)).

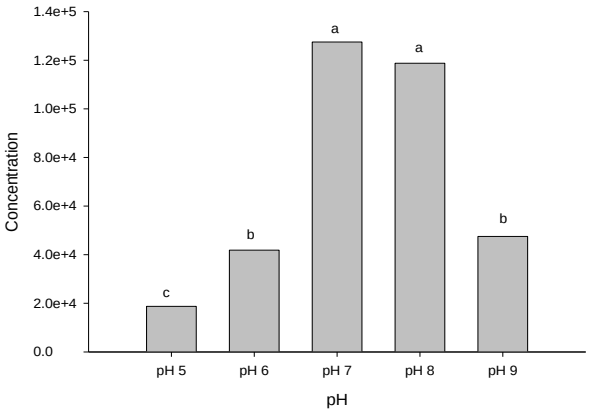


Figure 10: Influence of pH level on *C. cassiicola* sporulation incubated at 30°C on PDA-HS media.

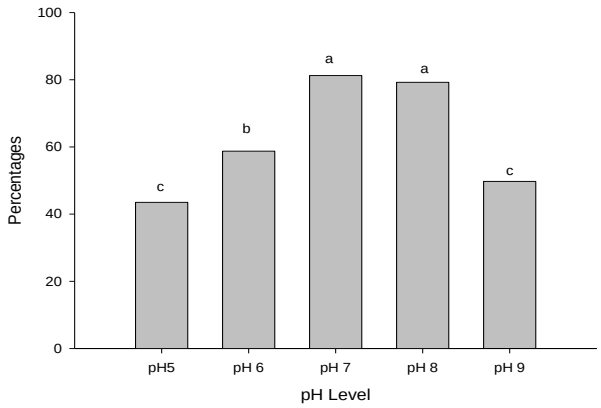


Figure 11: Influence of pH on *C. cassiicola* conidia germination incubated at 30°C on PDA-HS media.

DISCUSSION

It was critical to understand the factor that could provide an advantages and disadvantages to the targeted fungi (*C. cassiicola*) in order to get the best solution for the management of disease and their pathogen.

On the media suitability test, PDA-HS was the best media for C. cassiicola growth. The result was contradicted to the study by Nghia et al., 2008 which mentioned that the CMA was the best media for *C. cassiicola* growth and sporulation. It was also contradicted with study by Neni Kartini (1995) on V8 Agar which mentioned that best media for *C. cassiicola* growth was PDA. The result of this study showed both media mention was the lowest performance for the growth *of C. cassiicola*. The mycelium of *C. cassiicola* on CMA was also submerged into the agar made it impossible to produce a higher number of spores. In general, result of difference media was fulfilled the suggestion by different researcher which stated that the PDA was the best media for *C. cassiicola* growth Ahmed et al., 2013; Madhavi and Murthy, 2001). Other findings mentioned that the best media for *C. cassiicola* growth were PDA and V8J-Agar with no significant different (Neni Kartini, 1995).

The optimum temperature for the growth of *C. cassiicola* in this study was recorded at the range of 25°C to 30°C and similar result was reported by Ahmed et al., 2013b. At the 35°C treatment, the growth of *C. cassiicola* was clearly inhibited which was consistent with the previous results (Ahmed et al., 2013; Madhavi and Murthy, 2001). Temperature range of 25°C to 30°C was the optimum level for the sporulation and germination of *C. cassiicola.* (Ahmed et al., 2013; Madhavi and Murthy, 2001); and under 40°C temp, the conidia failed to germinate (Madhavi and Murthy, 2001) as also recorded in this study (Table 3).

Different density of light effect study was conducted on the growth, sporulation and spore germination of *C. cassiicola.* There was no significant different on the different light density on *C. cassiicola* growth. Meanwhile there was significant different on sporulation which 12HUV12HL was the best density of light for sporulation of *C. cassiicola* and the result was contradicted to the study done by (Celoto et al., 2015) which mention that the regime of light did not affect sporulation**.** Application of UV light could trigger *C. cassiicola* to produce many spore. Sporulation was greatest with 2h daily exposure to ultra-violet light.

Madhavi and Murthy (2001) mention that pH 5 to pH 7 was the best range for *C. cassiicola* growth with pH 6 was the optimum. Result of this study proved that pH range from pH7 to pH 8 was the best level of pH for *C. cassiicola* growth, spore production and spore germination. The lowest performance for *C. cassiicola* growth was at pH 5, the same goes to spore production. For spore germination pH 5 and pH 9 was the lowest percentage of spore germinates. The result was contradict to the study by Madhavi and Murthy (2001) which mentioned that pH 6 is the best price for spore germination after 8 hours. Rao and Mallaiah (1985) reported that maximum germination of *Cercospora conescens* was at pH5 and pH 7. Meanwhile the *C. cassiicola* was observed germinated at pH 5 to 9 (Madhavi and Murthy, 2001)

CONCLUSION

As a conclusion, temperature at 25°C was the best level for *C. cassiicola* growth. It could be the reason why the leaf spot disease increased during raining season as mention by most farmer interviewed at the early stage of this study. Most of the farmer mention they will have the problem weather in the farm or plant nursery during monsoon season normally by November to February every year. The spore recorded 30°C as optimum level which was represent the dry season in Malaysia between Aprils to September, a suitable time for conidia of *C. cassiicola* to be disseminated.

CONFLICT OF INTEREST

The authors declared that present study was performed in absence of any conflict of interest.

ACKNOWLEGEMENT
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AUTHOR CONTRIBUTIONS
MHS designed and performed the experiment. Other authors (NAB, SM, NN, ZS, BNMD, ZFAA, TAA and MMK) participated in writing every part of this manuscript and also all authors read and approved the final version.

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