Mycelia growth and sporulation of *Phytophthora colocasiae* isolates under selected conditions

ABSTRACT

This work was carried out at the International Institute of Tropical Agriculture (IITA), Yaoundé Nkolbisson. Ten improved and four local cultivars of taro were used to carry out a pathogenicity test of *Phytophthora colocasiae* isolates from which one virulent isolate from both improved cultivar BL/SM123, and BL/SM120 , were selected and subsequently used in determining the effect of media, temperature, pH, and light on growth parameters- mycelia growth and spores density. There was a significant difference ($P \leq 0.05$) on the growth media for the optimal fungal growth parameters. The most appropriate media for mycelia growth and spores production were V6 and V8 juice agar. The optimum temperature for mycelia growth and spore density was 24^0C and also optimum pH value for spores production was 6. The selection of the best growth media was important to screen the fungal pathogen for virulence and potential field pathogenicity testing.

Incubation in both light and dark was best for mycelia growth and sporulation.

Key words: taro cultivars, *Phytophthora colocasiae,* mycelia growth, sporulation.

INTRODUCTION

The major constraints of taro production in Cameroon are diseases and pests [1]. The crop is susceptible to fungal, bacterial, viral and nematode infections [2]. Among these various diseases, taro leaf blight disease is caused by *Phytophthora colocasiae* (Raciborski). It is one of the major important economic diseases of taro because it reduces corm yield of up to 50 % [3] and leaf yield of up to 95% in susceptible genotypes [4]. *Phytophthora colocasiae* causes corms to rot both in the field and in storage, and this has led to heavy storage lost [5]. In 2010 taro leaf blight disease was reported in Cameroon and it caused between 50-100 % yield loss of taro in most of the crop growing regions. This has led to a reduction in food, house hold income, increase poverty and some farmers have abandoned their farms and are now growing other crops [6, 7]..

Taro leaf blight disease (TLBD) is characterized by large necrotic zonated spots on the leaves often coalescing to destroy large areas of leaf [8]. The margin of the lesion is marked by a white powdery band of sporangia and numerous droplets of orange or reddish exudates [9].

Phytophthora colocasiae originated in South East Asia [8] and widely distributed throughout the tropical regions of the world [10].

Phytopthora colocasiae

Phytophthora colocasiae is primarily a foliar pathogen, but it also affects petioles and corms. The first symptoms on taro (*Colocasia esculenta*) are small, dark brown flecks or light brown spots on the upper leaf surface. These early spots often occur at the tips and edges of leaves where water accumulates [1, 10]. . They enlarge rapidly, becoming circular, zonate, and purplish-brown to brown in color. On the lower leaf surface, spots have a water-soaked, or dry gray appearance. As spots increase in size they coalesce and quickly destroy the leaf. In dry weather, or on some resistant cultivars, the centers of lesions become papery and fall out, producing a "shot-hole" appearance. Dead leaves often hang on their long petioles like flags. A prominent sign of *P. colocasiae* is the white ring of sporangia around the edge of lesions [2, 11].

Phytophthora species is composed of tube-like hyphae with few or no cross-walls (coenocytic), in contrast to the septate hyphae of fungi. Their nuclei are diploid (*2n*), compared to haploid (*n*), or dikaryotic (*n* + *n*) nuclei in the mycelia of most fungi. [3].

Asexual reproduction by *Phytophthora colocasiae*, ccurs during wet weather. Sporangia are formed at the end of short, unbranched or sparingly branched sporangiophores at the edge of lesions. They are ovoid to ellipsoid with a distinct narrow apical plug (semi-papillate), average 40-50 x 23 µm, and have a length-to-width ratio of 1.6: Sporangia are usually separated from sporangiophores by rain (caducous), leaving a stalk (pedicel) 3-10 µm in length attached to their base. [1]. During wet weather, sporangia germinate on the upper surface of leaves. When temperatures are near 20°C and humidity is high (90-100 %), most germination is indirect producing zoospores that swim for a few minutes, encyst, and form germ tubes. This process can occur in two hours or less. Sporangia germinate directly between 20-28° C, but may account for only a small percentage of total germination. The incubation period (time from germ tube penetration to development of symptoms) is 2-4 days at optimal temperatures of 24-27°C. [1, 12].

In order to understand the epidemiology of *Phytophthora colocasiae* and to obtain necessary information that could be useful for *in vitro* screening in the genetic improvement for resistance to taro

leaf blight disease, we studied the effect of media, temperature, pH, light on growth parameters- mycelia growth and spores density of the fungus.

Materials and methods

Four fungal isolates from two improved cultivars (BL/SM123, BL/SM120) and two local cultivars of taro were collected from the field at IITA Yaoundé. Using a pathogenicity test on these 4 host plants fungal virulence was identified based on their necrotic lesion production [11] and subsequently the most virulent isolate was used in determining the effect of media, temperature, pH, and light on growth parameters- mycelia growth and spores density of the fungus.

Effect of different culture media on the growth parameters of *P. Colocasiae*

Mycelial growth of the fungus

Culture media testing was aimed at selecting the best growth media for the fungus and the best mycelial growth of the fungus was used as a determinant in virulent strain selection for the pathogenicity test. The five culture media used were V8 juice agar, V6 juice agar, water agar, tomatoes-8- agar and potatoes dextrose agar (PDA). With the aid of a flame-sterilized 4 mm diameter cork- borer, mycelia discs were cut from an 8 day old axenic culture of *P. colocasiae* from two improved (BL/SM123, BL/SM120) and two local taro cultivars (Dark green petiole, White petiole). Each of the mycelia disc was aseptically transferred with the aid of a flame-sterilized mounted needle to the centre of different media tested. The bottom of the Petri dishes was marked by two perpendicular lines passing through the centre. Petri dishes was replicated four times for each cultivar and incubated at 24 ±2 ^{0}C at pH 6, and their mycelia growth was measured along the perpendicular lines using a ruler. The means of mycelia growth was then calculated from the different treatments on the 8th day [13].

Sporulation density of *P. colocasiae*

Culture media testing was used to select the best growth media for the fungus and the sporulation density used as a determinant in virulent strain selection for the pathogenicity test, Spore suspension was prepared from 21 days old culture of different isolates, by flooding the surface of the growing colonies in each Petri dish with 5 ml of sterile distilled water and dislodging the spores with a small brush. The suspension was centrifuged for 3 minutes and the supernatant was filtered through a 2 layered sterile muslin cheesed cloth. A drop of spore suspension was placed on the haemocytometer chamber, covered

with a slide and the number of spores per ml estimated as an average of the spores counted in 10 standard heamocytometer fields. The number of spores / ml was calculated using the formula adopted from Duncan and Torrance [12].

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Where S = Number of spores per milliliter

N = Mean number of spores in 10 large squares counted

V = 1 ml =1000 mm^3 *v*= volume of spore suspension under glass cover [13].

Effect of temperature on growth parameters of *P.colocasiae*

Four fungal isolates from the two improved cultivars and the two local cultivars were grown on V6 juice agar media. The most virulent isolate from the pathogenicity test was selected and incubated at temperatures of 15 ^{0}C, 17 ^{0}C, 24 ^{0}C and 33 ^{0}C with a constant pH of 6. Mycelia growth was measured with a ruler on the 8th day of incubation and sporulation density was measured on the 21st day of incubation using a haemocytometer [13].

Effect of pH value on growth parameters of *P. colocasiae*

The previous taro cultivars were cultured at different pH values ranges 4, 6, 7, 8 and 9 on V6 growth media and incubated at a temperature of 24± 2 ^{0}C. To prepare the different media 200 ml of V6 juice solution without agar was prepared and put in 5 conical flasks. The pH of the mixture was measured with the aid of electronic pH meter mark Thermo Orion. The pH of this mixture was 6.4 and two of the flasks content was adjusted by adding 10 % dilute hydrochloric acid progressively, until the required pH values (4 and 6) was observed. Ten percent of dilute sodium hydroxide (NaOH) was added to the other three mixtures to obtain the pH values of 7, 8 and 9. Four g of agar were added to each flask and well agitated. These mixtures were sterilized before adding antibiotics to prevent bacterial growth that could interfere with the study. From each of these media, 20 ml were placed in 5 Petri dishes per each cultivar. Four mm diameter fragment of mycelia obtained from an axenic culture was aseptically transferred with the aid of

the sterilized wire lobe and place at the center of solidified culture medium and incubated at the previous temperature. Two perpendicular lines were drawn at the bottom of the Petri dishes and Mycelia growth was measured with a graduated ruler on 8th day of incubation following the method of Fokunang *et al.* [13]. Data for sporulation density was recorded on the 21st day as described earlier.

Effect of light on growth parameters of *P. colocasiae*

Petri dishes with V6 juice agar were inoculated with 4mm diameter mycelia disc of an axenic cultures of the previous cultivars. Four Petri dishes of both local and improved cultivar were incubated in a dark cupboard and four placed under normal lighting condition for 21 days at pH 6 and temperatures of 24±2 ^{0}C. Mycelia growth was measured on the 8th day and spore density was determined on the 21st day,

Statistical analysis

Data on the effect of tested media, incubation temperatures, pH values tested and light on fungal growth parameters were subjected to analysis of Variance (ANOVA) as described by Wichura [14] using statistical software [15]. Mean variability amongst the cultivars were determined. Their treatment means were separated using Duncan Multiply Range Test (DMRT) and the Least Significant Difference (LSD) at statistical significance of 95% confidence interval.

RESULTS

Effect of culture media on the fungal growth parameter

Mycelial growth diameter of the fungus

Mycelia growth was observed on all the culture media 3 days after incubation for both the improved and the local cultivars as shown Table 1. The V8 juice agar and V6 juice agar showed a better performing growth media. Water agar showed the least mycelia growth in all the isolates, while the PDA and Tomato 8 agar showed moderate mycelial growth of the isolates. The surface of the media was covered with whitish mycelia 8 days after inoculation as shown in Figure 1. There was a significant difference ($p \leq 0.05$) in mean mycelia growth among the cultivars with culture media V8 Juice agar and Potatoes Dextrose Agar. The maximum mean mycelia growth diameter of 86.0±0.0 mm was observed in local cultivar white petiole withV6 Juice agar and V8

Juice agar media as opposed to minimum mean mycelia growth diameter of 27.7±1.7 mm observed in BL/ SM120, Dark green petiole and white petiole with water agar media after 8 days of incubation. Fungal growth performance was significant with culture media V6 juice and V8 juice Agar, respectively.

Table 1: Mycelia growth diameter (mm) of *P. colocasiae* 8 days after incubation on different culture media.

Cultivars	Mycelia growth diameter (mm)				
	V6 juice agar	V8 juice agar	PDA agar	Tomato 8 agar	Water agar
BL/SM 132	79.3±3.3a	70.0±5.8b	43.0±1.0ba	49.3±3.3a	29.3±1.7a
BL/SM120	80.7±2.9a	76.0±0.0ba	40.3±2.3b	51.0±2.9a	27.7±1.7a
Dark green petiole	82.7±3.3a	79.3±3.3ba	46.0±0.0a	39.3±13.3a	27.7±1.7a
White petiole	86.0±0.0a	86.0±0.0a	46.0±0.0a	52.7±13.3a	27.7±1.7a

123 Means followed by the same letters in the same column are not significantly different at $p \leq 0.05$ 124 (DMRT). Values are means of mycelia growth followed by standard error.

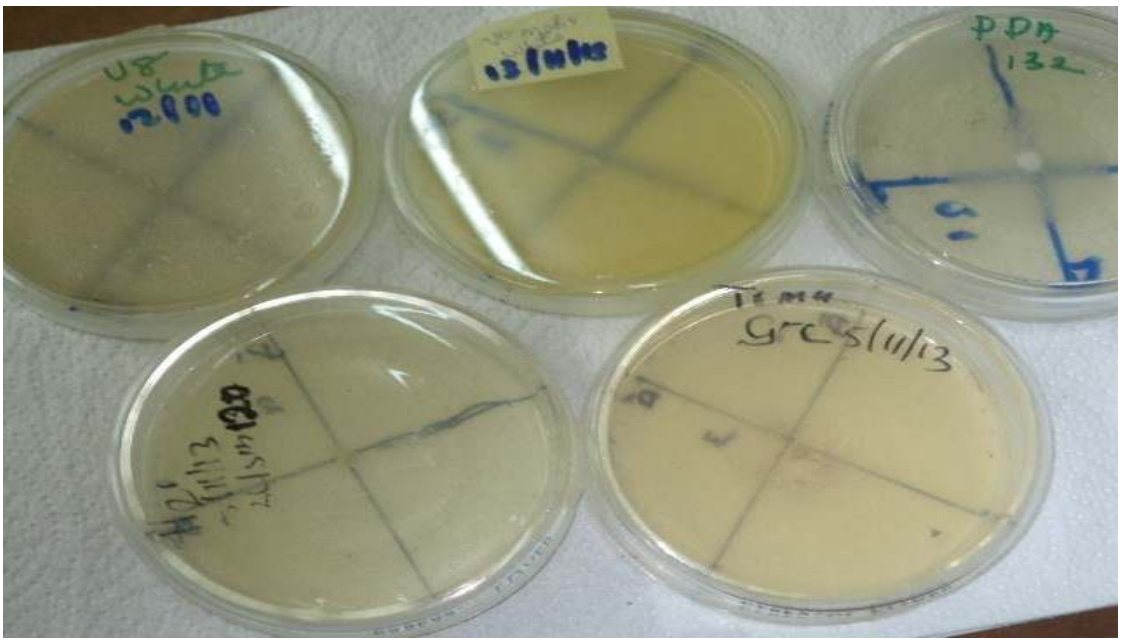

Figure 1: Growth of mycelia in different culture media.

Sporulation density of *P. colocasiae*

There was a significant variation in spore density with respect to the culture media as indicated in Table 2. Spores were not observed in all the cultivars with tomatoes and water agar media. There was sporulation in all cultivars with V6 Juice, V8 juice and potatoes dextrose agar at 21days after incubation. There was a significant difference ($p = 0.05$) in sporulation density among the cultivars with culture media V8 Juice agar and potatoes dextrose agar. A maximum mean sporulation density of 1.6×10^9 spores /ml of sterile distilled water was **observed** in local cultivar White petiole with V6 Juice agar media whereas **minimum** mean sporulation densities of $0.6\times 10^9$and 0.7×10^9 spores /ml of sterile distilled water were observed in BL/ SM132, Dark green petiole, with V8 Juice agar and potatoes dextrose agar media respectively, after 21days of incubation.

Table 2: Sporulation density of *P. colocasiae* 21 days after incubation on culture media

Cultivars	Sporulation value x (10^9 spores /ml)				
	V6 juice agar	**V8 juice agar**	**PDA agar**	**Tomato 8 agar**	**Water agar**
BL/SM 132	1.0±0.0a	0.6±0.1b	0.0±0.0b	0.0±0.0a	0.0±0.0a
BL/SM120	1.1±0.0a	0.8±0.0b	0.0±0.0b	0.0±0.0a	0.0±0.0a
Dark green petiole	1.5±0.1a	1.2±0.0a	0.7±0.3a	0..0±0.0a	0.0±0.0a
White petiole	1.6±0.0a	1.3±0.0a	1.0±0.0a	0.0±0.0a	0.0±0.0a

Means followed by the same letters in the same column are not significantly different at $p \leq 0.05$ (DMRT). Values are means spore density followed by standard error.

Effect of temperature on fungal growth parameters

Studies on the effect of temperature of growth parameters were conducted and the result showed that temperature variation has an influence of mycelial growth parameters as shown in Table 3. The four cultivars tested in V6 media had mycelia growth in all the different temperatures with excellent growth on all the cultivars at temperature of 24 ^{0}C. The highest mycelia growth of 73.3 mm at temperature of 24 ^{0}C was attained by cultivar BL/SM120 and white petiole. The least mycelia growth on all the cultivars was observed at temperature of 33^{0}C with cultivars BL/SM132 and BL/SM120 with 23.3±2.3 mm, 25.7±2.3mm, respectively. There was a significant difference ($p \leq 0.05$) of mycelia growth between the improved and local cultivars at temperature15 ^{0}C and 17 ^{0}C, respectively.

Table 3: Effect of temperature on mycelia growth diameter (mm) of *P. colocasiae* after 8 days of incubation.

Cultivars	Mycelia growth diameter (mm)			
	15^{0}C	**17^{0}C**	**24^{0}C**	**33^{0}C**
BL/SM 132	26.0±0.0b	46.0±0.0b	71.3±0.3a	23.3±2.3a
BL/SM120	26.0±0.0b	46.0±0.0b	73.3±0.7a	25.7±2.3a
Dark green petiole	32.7±1.7a	50.3±1.2a	72.3±0.9a	28.3±2.7a
White petiole	36.0±0.0a	50.3±0.3a	73.3±0.3a	33.0±4.0a

155 Means followed by the same letters in the same column are not significantly different at $p \leq 0.05$
156 (DMRT). Values are means of mycelia growth followed by standard error.

There was a significant difference ($p \leq 0.05$) in sporulation density among all the cultivars at a temperature of (17 ^{0}C and 24 ^{0}C , where dark green petiole cultivar had the highest sporulation density of 1.5×10^9 spores /ml on sterile distilled water at temperature of 24 ^{0}C (Table 4). The lowest sporulation (0.2×10^9 spores /ml) on sterile distilled water at 17 ^{0}C were recorded for BL/SM132and BL/SM120 cultivars. There was no sporulation of all the cultivars at temperature 15 ^{0}C and 33 ^{0}C respectively.

Table 4: Effect of temperature on sporulation of *P. colocasiae* after 21 days of incubation

Cultivars	Sporulation density x (10^9 spores /ml)			
	15^{0}C	**17^{0}C**	**24^{0}C**	**33^{0}C**
BL/SM 132	0.0±0.0a	0.2±0.0b	1.0±0.0b	0.0±0.0a
BL/SM120	0.0±0.0a	0.2±0.0b	1.1±0.0b	0.0±0.0a
Dark green petiole	0.0±0.0a	0.5±0.0a	1.5±0.1a	0.0±0.0a
White petiole	0.0±0.0a	0.5±0.0a	1.4±0.0	0.0±0.0a

Means followed by the same letters in the same column are not significantly different at $p \leq 0.05$ (DMRT). Values are means spore density followed by standard error.

Effect of pH on fungal growth parameter

Studies of the effect of pH on fungal growth parameters,(mycelial growth and sporulation) showed no significant variation in mycelial growth ($p \leq 0.05$) amongst the cultivars under incubation with the fungal isolates at pH values of 4, 6, and 7 as shown in Table 5. High mycelia growth was observed with all cultivars in all media. There was a significant difference ($p = 0.5$) in mycelia growth at pH values of 8 and 9. The lowest mycelia growth was recorded with all cultivars with a mean value of 66.0±0.0 mm at pH 4.

BL/SM120 and BL/SM132 with mean values of 84.67±0.7 mm at pH 7.

Table 5: Effect of pH on mycelia growth diameter (mm) of *P. colocasiae* after 8 days of incubation

Cultivars	Mycelia growth diameter (mm)				
	pH4	pH6	pH7	pH8	pH9
BL/SM 132	66.0±0.0a	74.7±2.9a	84.7±0.7a	70.0±0.0b	66.7±3.3b
BL/SM120	66.0±0.0a	76.7±3.3a	84.7±0.7a	82.0±2.0a	73.3±3.3ab
Dark green petiole	66.0±0.0a	81.7±4.3a	85.3±0.7a	81.0±0.0a	77.3±3.7a
White petiole	66.0±0.0a	81.0±5.0a	85.7±0.3a	81.0±0.0a	81.0±0.0a

Means followed by the same letters in the same column are not significantly different at $p \leq 0.05$ (DMRT). Values are means of mycelia growth followed by standard error.

There was significant difference ($p \leq 0.05$) in spore density at pH values of 7 and 8 among the local and improved cultivars (Table 6). Spores were not observed at pH 4 and 9 in all the cultivars. Cultivars dark green petiole and white petiole had high sporulation density with mean values of $1.2\pm0.5\times 10^9$ and $1.5\pm0.0\times 10^9$ spores /ml of sterile distilled water at pH 6, respectively. The lowest sporulation density values were recorded with cultivars BL/SM 132 and BL/SM120 with mean value of $0.1\pm0.0\times 10^9$ spores /ml of sterile distilled water at pH 8.

Table 6: Effect of pH on sporulation density of *P. colocasiae* after 21 days of incubation

Cultivars	Sporulation density x (10^9 spores /ml)				
	pH4	pH6	pH7	pH8	pH9
BL/SM 132	0.0±0.0a	1.0±0.6a	0.4±0.1b	0.1±0.0b	0.0±0.0a
BL/SM120	0.0±0.0a	1.1±0.0a	0.5±0.0b	0.1±0.0b	0.0±0.0a
Dark green petiole	0.0±0.0a	1.2±0.5a	1.1±0.7a	0.4±0.0a	0.0±0.0a
White petiole	0.0±0.0a	1.5±0.0a	1.1±0.1a	0.4±0.0a	0.0±0.0a

Means followed by the same letters in the same column are not significantly different at $p \leq 0.05$ (DMRT). Values are means spore density followed by standard error.

Effect of light on fungal growth parameter

There was no significant difference in mycelia growth in light and dark condition among the cultivars. High mycelia growth was observed with all the cultivars in both light and dark exposure conditions. (Table 7).

Table 7: Effect of light on mycelia growth diameter (mm) of *P. Colocasiae* after 8 days of incubation

Cultivars	Exposure conditions	
	Light	Dark
BL/SM 132	72.0±0.0a	75.3±5.3a
BL/SM 120	76.6±4.8a	78.7±4.7a
Dark green petiole	72.3±0.8a	77.3±4.7a
White petiole	73.3±0.3a	82.7±3.3a

Means followed by the same letters in the same column are not significantly different at p = 0.05 (DMRT). Values are means of mycelia growth followed by standard error.

There was a significant difference (p = 0.5) in sporulation density in light and dark among the cultivars. High sporulation density of 1.5±0.0 was observed with cultivar white petiole and dark green petiole in light and low sporulation density of 0.9±0.3 was observed with cultivar BL/SM 132 in dark exposure conditions (Table 8).

Table 8: Effect of light on sporulation density x (10^9 spores /ml) of *P. colocasiae* after 21 days of incubation.

Cultivars Exposure conditions		
	Light	**Dark**
BL/SM 132	1.0±0.0c	0.9±0.0b
BL/SM 120	1.2±1.3b	1.0±0.0b

Dark green petiole	1.5±0.0a	1.4±0.0a
White petiole	1.50±0a	1.4±0.0a

Means followed by the same letters in the same column are not significantly different at p = 0.05 (DMRT). Values are means spore density followed by standard error.

Discussion

From the studies carried out on two improved and two local cultivars BL/SM132, BL/SM120, dark green petiole and white petiole, mycelia growth was observed in all the media with maximum mycelia growth observed in V6 and V8 juice agar media as opposed to poor mycelia growth in water and tomatoes agar media. Growth media has an important role in the growth potential and virulence studies of fungi. In a suitable growth condition most pathogenic fungi show maximum growth potential and virulence on their host crops [10]. The potential growth media is in accordance with results of Tsopmbeng et *al*. [16] who stated that both V6 and V8 Juice agar media were the most suitable for *Phytophthora colocasiae* cultivation in Cameroon. The poor development of fungus on water and tomatoes agar medium may be due to its nutrient. Nutrient is very important in the expression of the fungus in the culture media. The absence of spores and slow mycelia growth in water and tomatoes culture media could be due to the absence of some minerals which were necessary for the growth and development of the fungus. The most appropriate media where spores were produced were V6 and V8 juice agar, with little spores produced on local cultivar in potatoes dextrose agar. Growth media have been shown to influence spore germination, and other growth parameters in the virulent fungi [13]. The study of growth media is therefore important for the selection of growth media specific and favourable for the culture and growth of *P colocasiae*.

Optimum sporulation and mycelia growth was observed at temperature 24 ^{0}C in all the cultivars. This result agree with the works of Fullerton and Tyson [17] who reported that the optimum temperature for growth *in vitro* is approximately 25 ^{0}C in detached leaf tissues, the rate of symptom development is greatest at temperatures 25-30 ^{0}C and at 35 ^{0}C symptom development is halted. Under optimum conditions (relative humidity approaching 100 %, temperatures ranges of 20-25 ^{0}C) sporulation can take

place at the margin of lesion in less than 3 hours. This study had shown that temperature at 24 ^{0}C was the best for mycelia growth and sporulation.

As concerns pH, there was high mycelia growth on all the cultivars in all the pH media both acidic and basic. Spores were not produced at very low pH 4 (highly acidic) and at very high pH 9 (highly basic). High sporulation density 1.5× 10^9 spores /ml of sterile distilled water were observed at pH 6. This was in accordance with report by Sahu *et al.* [18] who stated that pH 6, 5 and temperature of 28 ^{0}C is favorable for the growth of *P. colocasiae.*

Results on light showed that there was no effect on sporulation and mycelia growth because there were spores and mycelia growth in all the cultivars both improved and local. The selection of the best culture growth medium is important for maximum growth and sporulation of the fungus. A good nutritive media is important for fungal mycelial growth, sporulation and spore germination necessary for the virulence and pathogenicity growth [7]. Fungal virulence is important for disease incidence and severity especially with a favourable growth condition.

Conclusion

The assessment of growth parameters on culture media, and the effect of temperature, pH and light on P colocasiae has confirmed that culture media, temperature and pH had a great influence on mycelia growth and sporulation density but light had no impact. Mycelia growth and sporulation density were dependent on the growth medium. The most appropriate media for mycelia growth and spores production were V6 and V8 juice agar. The most suitable temperature for mycelia growth and spore density was 24 ^{0}C and also the best pH for spores to be produced was 6. The selection of the best growth media is important to screen the fungal pathogen for virulence and potential field pathogenicity testing.

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