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Growth and physiological studies on root rots fungus of cowpea

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ABSTRACT

Growth and physiological studies on Pythium aphanidermatum was carried out. The mycelia of the fungus grew satisfactorily on the entire agar media used. Potato Dextrose Agar (PDA) was found to be the best medium in term of mycelial extension, mycelial density and sporangia production compared to Malt extract and Czapeck-Dox agar. The best mycelial growth of Pythium aphanidermatum was on potato dextrose agar (PDA). The optimum temperature for the mycelial growth of the fungus was observed at 30^{o}C and sporangia production was favoured at 25^{o}C. The results of investigations on the effect of temperature on the sporangia production of P. aphanidermatum showed that sporangia production and germination was best at 30^{o}C. Sporangia production was poor at 25^{o}C and 35^{o}C. There was no sporangia production at 20^{o}C and 40^{o}C. And on the effect of relative humidity on mycelia growth and sporangia formation, the result showed that P. aphanidermatum grew and produced sporangia much more profusely at high relative humidity than at low relative humidity level when incubated at 27^{o}C. The highest rate of sporangia production (15.33×10^{4} sporangia) occurred at 100% relative humidity, while no sporangia were observed to occur at relative humidity levels lower than 32.5%. Amongst the carbon sources supplemented, starch was best utilized with the highest mycelia dry weight of 0.74g /30ml media but glucose favoured sporangia production. Urea was most utilized among the nitrogen sources with the highest number of sporangia production.

Keywords: Growth, Physiology, humidity, Carbon, nitrogen, temperature.

INTRODUCTION

Fungal diseases constitute the major limiting factor to the production of cowpea in Nigeria. Losses caused by fungi vary from 20 to almost 40 percent. *Pythium* sp. is the major source of

root rot disease of cowpea in Kogi East Senatorial district. It also causes rot and damping off of C*orchorus olitorius* and *Lycopersicon esculentum* respectively in the area. *Pythium* is a pathogenic fungus belonging to the family Pythiaceae. It usually attack seedlings at the base and root under conditions of overcrowding and over-watering [3]. They are members of oomycetes or water molds. Some require specific temperature optima: *P. ultimum* relatively low, 20^{o}C; *P. aphanidermatum* up to 35^{o}C. *Pythium* fungus cause soft, watery rots of seeds, roots, storage organs and crown tissues. Their hyphae are hyaline and aseptate (not containing cross walls) [7]. [3] reported *P. debaryannum* as the most common species and when attacked, the seedlings become weakened at the base and soon fall off or die off. [15], reported *Pythium aphanidermatum* as one of the major pathogen of cowpea, which is widely distributed in Nigeria where cowpea is extensively grown. *Pythium aphanidermatum* has been reported in Japan, India, Australia, South Korea and Taiwan as a serious cowpea pathogen [5].

Pythium is a pathogenic fungus and affected by different growth media like any other pathogenic fungus. [12], while working on growth requirements of *Alternaria solani* affecting tomato, reported that the fungus grows more on Potato Dextrose Agar (PDA) than any other growth medium. On sporangia production, they reported that inocula obtained from primary Potato Dextrose Agar (PDA) and malt agar (MA) culture did produce more spores than on other cultures.

On temperature requirements for the growth and sporangia production of this fungus, [7], reported that *P. aphanidermatum* and *P. ultimum* require specific temperature optima of 35^{0}C and 20^{0}C respectively. It is a known fact that different carbon and nitrogen sources are required by fungi for their physiological development. [11], while studying the factors affecting the occurrence and severity of blackmold disease reported that water soluble nutrients of glucose and fructose on the fruit surface dissolved in the dew, stimulated germination of conidia. They also reported that the requirement of the causal fungus with regards to carbon sources varies with concentrations of each source. According to them, glucose, maltose, fructose at concentrations 21.62mg/litre have been shown to be superior in supporting the growth. [13], reported calcium nitrate being utilized by *Alternaria* than other nitrogen sources. Growth media have been known to have an impact on fungi. Also, the relevance of carbon and nitrogen sources on the development of fungi has been stressed. This research was therefore undertaken in order to establish the physiologically relevant effects and impacts of media, nitrogen and carbon sources as well as the temperature requirement on the growth and physiological development of this fungus as agent of root rots of cowpea.

MATERIALS AND METHODS

Effect of potato dextrose agar, czapeck-dox agar and corn meal agar media on the linear growth, sporangial production and germination of isolate.

Five millimetres -discs of 7 day- old agar culture of the fungus was inoculated on agar media in petridishes and incubated at 27$\pm$ 2^{o}C for five days. Two perpendicular lines intersecting at right angles were drawn at the bottom of each plate. A 5mm diameter disc, of the test organism was cut with a sterile cork borer and placed on a freshly prepared solid medium in the plate at the intersect of the two lines. The radial growth of the tests organism was measured at 24- hour intervals. By subtracting 1 cm (initial radius of inoculum) from the overall radial growth

measurement and dividing by the number of days of incubation, the growth rate per day (in cm.) was obtained. The mean of three replicated plates per medium was divided by the number of days, this gives the average growth rate per day and considered as the growth rate per observation by methods of [4].

Effect of temperature and relative humidity on the linear growth, sporangial production and germination of the isolate on potato dextrose agar.

Experiments on temperature relations were conducted in incubators set at the following temperatures: 20±2^{o}C, 25±2^{o}C, 30±2^{o}C, 35±2^{o}C and 40±2^{o}C. Five millimetres mycelia blocks from seven-day old agar cultures were inoculated and incubated in incubators set at the above temperatures. The mean of three replicate plates per temperature was considered as the growth value per observation by methods of [4], as described in section above. Varying levels of relative humidity were obtained above saturated solutions of some salts in desiccators. The salt solutions used and their corresponding relative humidities are as follows: P_2O_5, 0%; $MgCl_2.6H_2O$, 32.5%; Glucose, 55%; NaCl, 75%; KCl, 85%. Distilled water gave 100% relative humidity.

Effect of different carbon sources on mycelial dry weight and sporangial production of the isolate.

This was determined by dry weight method. Mycelial dry weight determination was carried out on mycelial growing on different carbon sources in a liquid medium following the method of [13]. The following carbon sources were incorporated into basal medium; glucose, starch and fructose. The basal medium consisted of: 1.0g, KCL; 0.5g, Mg SO_4 $7H_2O$; 3.0g, Ca $(NO_3)_2$; 1.0g, K_2HPO_4; 0.01g, $FeSO_4$ $7H_2O$. The appropriate weight of each carbon source (10g dissolved into 100ml distilled water) was autoclaved separately; 20ml of the basal medium were dispensed into conical flask and autoclaved at 121^{o}C for 15 minutes. Ten (10ml) solutions of the different sugars (sterile) were aseptically added to the sterile basal medium in the flasks. Each conical flask contained 20ml sterile basal medium and 10 ml sterile carbon source. The flasks were inoculated with 5mm diameter disc of test fungus of a seven-day old culture growing on PDA. Three replicates flasks were used for each carbon source. The mycelia were filtered by suction through filter paper previously dried to a constant weight. Both filter paper and mycelia were then dried in an oven at 80^{o}C to a constant weight on whatman's filter paper. The weight of the mycelia was determined by subtracting the initial weight of the filter paper from the weight of mycelia and filter paper.

Effect of different nitrogen sources on mycelial dry weight and sporangial production of the isolate.

Similar procedure as above was carried out with liquid medium. The appropriate weight of each nitrogen source was autoclaved separately before adding aseptically to the basal medium in the flasks. The different nitrogen sources used were ammonium chloride (NH_4Cl), sodium nitrate ($NaNO_3$), urea [C $(NH_2)_2$ and calcium nitrate Ca $(NO_3)_2$. Ten (10ml) solutions of the different nitrogen (sterile) were aseptically added to the sterile basal medium in the flasks. Each conical flask contained 20ml sterile basal medium and 10ml sterile nitrogen source. The flasks were inoculated with 5mm diameter disc of test fungus of a seven day old culture growing on PDA such that the mycelial matt were uppermost and floated on the medium. The experiment was replicated. The flasks were stoppered with sterile non – absorbent cotton wool and incubated at room temperature for one week at 25 ± 2^{o}C. Harvesting was carried out at five days interval.

The mycelia were filtered by suction through filter paper previously dried to a constant weight. Both filter paper and mycelia were then dried in an oven at 80^{o}C to a constant weight on whatman's filter paper. The weight of the mycelia was determined by subtracting the initial weight of the filter paper from the weight of mycelia and filter paper.

RESULTS

Growth studies of *Pythium aphanidermatum* on different media.

The mycelia of *Pythium aphanidermatum* grew satisfactorily on the agar media used. However, the level of growth recorded varied with different media used. The best and the fastest growth values were recorded on potato dextrose agar (PDA) of average growth rate (2.65cm/day), where the surface of the medium was completely covered at the end of the fifth day after inoculation. Apart from mycelial extension, potato dextrose agar was equally the best medium in terms of mycelia density, sporangial production and germination compared with malt extract (MEA) and Czapeck-dox agar (CDA). This was closely followed by Czapeck-dox agar of average growth rate (2.40cm/day). Malt extract agar was the least in terms of mycelial extension with average mycelial growth rate of 2.12cm/day, mycelial density and no sporangial production. Sporangial production was poor or totally absent on the media, using the two inoculation techniques described under materials and methods. Mycelia transfer technique failed to induce sporangia formation on any of the media tested whereas there was sporangia formation involving sporangia flooding transfer methods. No sporangia formation occurred on malt extract agar. Out of the fifty sporangia produced in PDA, only ten germinated; while only four germinated in CDA out of thirty-one produced. However, there was no significant difference among the media at 5% in terms of mycelia growth.

Effects of temperature and relative humidity on mycelial growth and sporangial production in *Pythium aphanidermatum* on potato dextrose agar.

The highest radial growth was observed at 30^{o}C. Mycelia growth at this temperature was significantly better than at other levels of temperature tested at 0.05% level of significance compared with other levels of temperature; there was no significant difference at 25^{o}C and 35^{o}C at (P 0.99 > 0.05), also no significant difference between 20^{o}C and 40^{o}C at (P 1.00 > 0.05) when compared **(Table 1)**. There was slow growth at 25^{o}C while there was no appreciable growth at 20^{o}C and 40^{o}C. It was also observed that all the levels of temperature that supported radial growth also supported sporangia production and sporangia germination. The results of investigations on the effect of temperature on the sporangia production of *P. aphanidermatum* showed that sporangia production and germination was best at 30^{o}C. Sporangia production was poor at 25^{o}C and 35^{o}C. There was no sporangia production at 20^{o}C and 40^{o}C. There was, however, no difference in the morphology or the dimension of the sporangia formed at the different temperature levels. And on the effect of relative humidity on mycelia growth and sporangia formation, the result showed that *P. aphanidermatum* grew and produced sporangia much more profusely at high relative humidity than at low relative humidity level when incubated at 27^{o}C **(Table 2)**. The highest rate of sporangia production (15.33×10^{4} sporangia) occurred at 100% relative humidity, while no sporangia were observed to occur at relative humidity levels lower than 32.5%.

Table 1: Effects of temperature on mycelial growth and sporangial formation and germination of *P. aphanidermatum*

Temperature levels (^{o}C)	Mean mycelial growth± SE(cm)	Mean sporangia No. ± SE (×10^4)	Mean sporangia germination ± SE (×10^4)
20	0.00±0.00 ^{a}	0.00±0.0 ^{a}	0.00±0.0 ^{a}
25	1.49±0.11 ^{b}	19.33±0.3 ^{c}	1.33±0.3 ^{c}
30	2.67±0.36 ^{c}	48.00±0.6 ^{d}	15.33±0.3 ^{d}
35	1.40±0.88 ^{b}	15.00±0.0 ^{b}	3.00±0.0 ^{b}
40	0.00±0.00 ^{a}	0.00±0.0 ^{a}	0.00±0.0 ^{a}

Means represented by the same letter are not significant different (P ≤ 0.05).

Table 2: Effects of Relative Humidity (RH) on sporangial production and germination of *Pythium aphanidermatum*.
Results are means of three replicates ± S.E.

Relative Humidity (%)	Number of sporangia (×10^4)	Number of sporangia germinated (×10^4)
32.5	2.33 ± 0.3 ^{a}	0.00± 0.0 ^{a}
55	8.00 ± 0.0 ^{b}	0.00± 0.0 ^{a}
75	8.33 ± 0.3 ^{b}	0.00± 0.0 ^{a}
85	12.00 ±0.0 ^{c}	3.00± 0.0 ^{b}
100	15.33±0.3 ^{d}	5.00±0.0 ^{b}

Means represented by the same letter are not significantly different (P ≤ 0.05).

Effects of carbon sources on the mycelial dry weight and sporangial production of *Pythium aphanidermatum* incubated for fifteen days.

Glucose, fructose and starch were supplemented, and growth was determined by dry weight method. **Table 3** shows the effect of different carbon sources on the mycelia growth. The highest mean mycelia dry weight (740mg) was recorded on starch; followed by fructose (360mg), while glucose had the least (320mg). The results showed that *Pythium* utilized starch better than other carbon sources tested. With HSD of 4.04g, there was a significant difference between the control and starch at (P 0.04 < 0.05). There was no significant difference among the carbon sources incorporated (P ≤ 0.05). Statistically, there was a significant difference in the carbon sources compared with control. Sporangia production and germination were more in glucose compared to starch and fructose.

Effects of nitrogen sources on mycelial dry weight and sporangial production of *Pythium aphanidermatum* incubated for fifteen days.

Urea was the best nitrogen source with the highest mean mycelial dry weight of 670mg, there was a significant difference (P 0.01 < 0.05) compared with the control (120mg); this was closely followed by ammonium chloride with mean mycelia dry weight of 460mg; while sodium nitrate and calcium nitrate had 390mg and 360mg respectively, with no significant difference among the nitrogen sources supplemented at P ≤ 0.05%. Sporangia production and germination also varied among the nitrogen sources. There was low production of sporangia in calcium nitrate (56) and sodium nitrate (59). Urea and ammonium chloride produced abundant sporangia (64 and 61) but germination was more in ammonium chloride **(Table 4)**.

Table 3: Mean (± S.E) mycelial dry weight and sporangial production of *Pythium aphanidermatum* on different carbon sources

Carbon sources	Mycelial dry weight (g)	Number of sporangia ($\times 10^4$)
Control (basal medium)	0.12 ± 0.4 [a]	50 ± 1 [a]
Glucose	0.32 ± 0.1 [a b]	63 ± 0 [b]
Fructose	0.36 ± 0.1 [a b]	57 ± 1 [a]
Starch	0.74 ±0.2 [b]	59 ± 2 [a]

Means represented by the same letter are not significantly different (P ≤ 0.05).

Table 4: Mean (± S.E) mycelial dry weight and sporangial production of *Pythium aphanidermatum* on different nitrogen sources

Nitrogen sources	Mycelial dry weight (g)	Number of sporangia ($\times 10^4$)
Control (basal medium)	0.12 ± 0.0 [a]	56 ± 0 [a]
Calcium nitrate	0.36 ± 0.0 [a b]	56 ±1 [a]
Sodium nitrate	0.39 ± 0.1 [a b]	59 ± 1 [a]
Ammonium chloride	0.49 ± 0.1 [a b]	61 ± 0 [a]
Urea	0.67 ± 0.1 [b]	64 ± 1 [b]

Means represented by the same letter are not significantly different (P ≤ 0.05).

DISCUSSION

The isolated fungus associated with root rot of cowpea which was identified as *Pythium aphanidermatum* had all the morphological features of *Pythium* described by [8] and [1]. There have been several conflicting reports on the best media, which support the growth of *Pythium aphanidermatum.* Among the three media for comparative growth of *P.aphanidermatum*, [5] found CMA to be most suited for linear growth and sporangial production. The present comparative studies on the radial growth of *P. aphanidermatum* on three agar revealed that the fungus generally grew slowly and differently on all the solid media tested. The study showed that potato dextrose agar supports fastest growth and sporangial production and germination; this is in agreement with [5] where they reported that *P. vignae* and *P. aphanidermatum* grew well on CMA and PDA. This is also in agreement with [12] that *Alternaria solani* grew more in PDA than other growth media. And on sporulation, they found that inocula obtained from primary PDA and MA cultures did produce more spores than other cultures and except for water agar, rye agar produced the lowest spore yields. It is a known fact that there is effect of water potential and media on production and sporangial germination by fungi. [14] found that formation of sporangia in *Pythium* was maximum on either potato dextrose agar or water agar containing carnation leaf pieces and adjusted to different water potentials. In this study, the best medium in terms of mycelial density and sporangia production and germination is in PDA.

The optimum temperature for growth and sporangia production of this fungus showed that this fungus grew well between 25±2^{o}C and 35±2^{o}C with optimum growth at 30±2^{o}C, which incidentally is the daily temperature during the cowpea's growing season (Meteorological Station, Geography department, Kogi State University, Anyigba). This observation had earlier been reported by [10] that root rot of cowpea incited by *Pythium aphanidermatum* is optimum at about 30±2^{o}C. The optimum temperature for the formation and germination of sporangia was however found to be 25±2^{o}C. It is possible that processes such as germ-tube formation, appresorium formation or host penetration respond to temperature, hence the above result. [13]

reported the effect of temperature on germination of sporangia or spores; which largely determine the limit within which the response of infection of any disease can occur because sporangia which do not germinate cannot infect the host in some pathogenic fungi. The various optimal temperatures for mycelial growth, sporangial production and germination of *P. aphanidermatum* obtained in the laboratory coincide favourably with the commonly observed field temperatures. This may therefore not only account for the survival of the pathogen in the field (soil), but also enhances disease incitement and development.

High atmospheric humidity has been reported to favour the initiation of the diseases of many plants [2]. In this study the severity of the disease, sporangial production and germination of *P. aphanidermatum* were found to be greatly enhanced by high relative humidity, rainfall and high temperature. This may account for the high incidence and severity recorded for the disease in warm moist periods of the growth seasons. The results have been correlated with climatological data. Rainfall and relative humidity are normally highest between July and September coupled with optimum temperature and less sunshine hours, this determines whether there are adequate period of wetness for rot infection and subsequent disease establishment.

The results on physiological studies showed that growth was supported by all the carbon sources tested. Starch supported the highest mycelial dry weight; while sporangial production was more in glucose, this is in agreement with [6] who reported that growth and sporangial production of *Pythium* were highest on soluble starch. [11], studied the factors affecting the occurrence and severity of Blackmold of Ripe Tomato Fruit and found that water soluble nutrient such as glucose and fructose, on the fruit surface dissolved in the dew, stimulated germination of conidia. In the present study, glucose and fructose were utilized with an average mycelia dry weight (320mg/30ml) media and 360mg/30ml media respectively at the end of the fifteen days of inoculation. The highest mean dry weight (740mg) was recorded in starch. The results showed that *Pythium* utilized starch better than other carbon sources. With HSD of 4.04g, there was a significant difference between the control and starch at (P 0.04 < 0.05). This was closely followed by fructose. There was no significant difference among the carbon sources incorporated ($P \leq 0.05\%$), but statistically, there was significant difference in the carbon sources compared with control. Sporangial production and germination was more in glucose compared to starch and fructose.

On nitrogen utilisation, it has been reported that the level as well as the nature of the nitrogen, an essential element used for both physiological as well as for morphological purposes are of vital importance in determining fungal development. Likewise different fungi show differential ability in utilizing different nitrogen sources [13]. The results of the present study on four nitrogen sources namely, ammonium chloride (NH_4Cl), urea [C $(NH_2)_2$ calcium nitrate [Ca $(NO_{3)2}$] and sodium nitrate ($NaNO_{3)}$ showed that *Pythium* utilized urea better than other nitrogen sources. Calcium nitrate and sodium nitrate appeared to be poor nitrogen sources for the growth of *Pythium*. The best nitrogen source was urea with the highest mean mycelia dry weight of 670mg, there was significant difference (P 0.01<0.05) compared with the control; this was closely followed by ammonium chloride with mean mycelia dry weight of 460mg; while sodium nitrate and calcium nitrate had 390mg and 360mg respectively, with no significant difference among the nitrogen sources supplemented at $P \leq 0.05\%$. Sporangial production and germination also varied among the nitrogen sources. There was low production of sporangia in calcium nitrate and

sodium nitrate. Urea and ammonium chloride produced abundant sporangia but germination was more in ammonium chloride. The highest number of sporangia production was however obtained in urea.

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