THE ECONOMIC IMPORTANCE OF FUNGICIDE CONTROLLED STUDIES ON *PYTHIUM APHANIDERMATUM ISOLATED* FROM COWPEA ROOT ROTS

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ABSTRACT

The economic losses of cowpea due to fungal attack were reported to be alarming in the study areas. Laboratory studies were carried out to isolate, identify and chemically control the fungus associated with root rot of cowpea (*Vigna unguiculata* (L.) Walp. From samples, two methods of fungal isolation (serial dilution from soil sample and direct plating of infected root) were used. *Pythium aphanidermatum* was isolated and identified. Benlate (Benomyl), Mancozeb and Ridomil, being fungicides applied, controlled the mycelial growth of the fungus *in vitro*. The percentage infection reduction range from 6.7% to 33.3% among fungicides employed, suggesting an economic gain. The *in vitro* bioassay of these fungicides showed that Benlate and Mancozeb inhibited linear growth of the fungus at 150 ppm and 200 ppm and active ingredient of 50% and 80% respectively, while Ridomil was most effective at 40 ppm with active ingredient of 66%. These fungicides equally exhibited inhibitory effect on sporangia production, germination and mycelial dry weight of the fungus. In view of the positive results, these fungicides, with collaborative research among all stake holders, will provide an integrated method of managing the disease.

Key words: Isolation, in *vitro, Pythium,* fungicides, mycelium

INTRODUCTION

Cowpea, *Vigna unguiculata* (L.) Walp of the family Fabaceae(Leguminosae); is a tropical, herbaceous annual plant, which is wide spread all over the world. It is of major importance to the livelihood of millions of relatively poor people in developing countries of the world.

A large number and diversity of cultivated varieties of cowpea throughout Africa and over the Southern half of Asia and adjacent lands as well as the Mediterranean region of Europe indicate that the area is of ancient cultivation of cowpea as human food. Cowpea has been cultivated in Africa since prehistoric times. It is believed that cowpea reached South Western Asia about 2300 BC and came to Southern Europe early enough for the Greeks and Romans to grow it under the name of *Phaseolos* (Purseglove, 1974). Cowpea was introduced into the new world in the late seventeenth century by the Spanish and more cultivars were transported there from East Africa with the slave trade into Ethiopia, Central Africa and later West Africa. Rawal (1975) stated that cowpea might have originated in West Africa, very likely Nigeria, where a profusion of wild and weedy species in the Savannah and forested zones, still exist. It is an herbaceous, short duration annual grain legume and broadly adapted and capable of seed production in semi arid regions, where the amount and distribution of rain is not adequate for other legumes and in

the strongly leached ultisols and oxisols of the humid tropics. Cowpea is now grown throughout the tropics and subtropics and has a wide variety of uses including hay, grain, and green manure and also as a vegetable (Rachie and Rawal, 1976).

In Nigeria, cowpea is the most important indigenous grain legume found in most areas north of the confluence of Rivers Niger and Benue. Cowpea is grown in almost every ecological zone except in the mangrove swamps. It is estimated that over 80% of the total cowpea produced in Nigeria are grown north of latitude10°N (Savannah and derived savannah belts) including Kogi State, where it traditionally in admixture with other crops (Williams, 1975). Out of the estimated 12.5 million hectares of land grown with cowpea and annual production of over 3 million tonnes world-wide, substantial part of this production comes from Nigeria (about 4 million hectares of land, with 1.7 million tonnes of grain (Singh, *et al.,* 1997).

The most devastating diseases of cowpea are fungal diseases. Many fungi have been identified by various researchers as causal organisms of various cowpea diseases. According to Williams (1975), the major fungal diseases of cowpea in Nigeria include seedling mortality caused by *Pythium aphanidermatum* (Edson) Fitzp.) and *Corticium solani* (Prill. & Delact.). Others are authracnose caused by *Collectotrichum lindermuthianum* (Sacc. & Magn.), cercospora leaf spots caused by *Cercospora canescens* and *C. cruenta*, rust caused by *Uromyces appendiculatum.* Brown rust caused by *Uromyces appendiculatum*, while Onuh *et al* (2005) reported brown rust caused by *Uromyces appendiculatum* as the most important fungal disease of cowpea in Florida, U.S.A. Emechebe and Shoyinka (1985) regarded cowpea rust as a major cowpea disease in the rainforest and Southern Guinea Savanna Zone of West Africa and in medium- elevation area of East Africa. Brown Blotch caused by *C. truncatum* was reported by Gillian (2006) and Croft (2007). Singh and Allen (1979) reported thirty – five diseases of cowpea most of which are caused by fungi.

The two most important destructive fungal diseases of cowpea in the Guinea Savanna Zone of Nigeria are scab disease caused by *Sphaceloma* Sp. and brown blotch induced by *Colletotrichum capsici* (syd.) (Emechebe and Shoyinka, 1985). This was recently corroborated by Gillian (2006) and Croft (2007). Scab was reported from Southern Nigeria at IITA, Ibadan in 1990. *Septoria* leaf spot caused by *Septoria vignae* occurred in the Guinea Savanna as reported by (Emechebe, 1980). Moving towards the Sudan Savanna, Macrophomina blight of cowpea induced by *Macrophomina phaseolina* is of major concern while basal stem rot induced by *Fusarium solani* is of minor importance in this zone (Emechebe and Shoyinka, 1985). Hagan, (2001) and Adeyeye and Olufolaji, (2004) have separately reported ascochyta blight caused by *Ascochyta phaseolorum* Scc., as one of the major cowpea diseases in Africa, which occurs more frequently in the hot, rainy season than in the dry season. Root rot disease of cowpea is one of the major diseases of cowpea in Kogi State; whose effective management is crucial to cowpea production in the area. A survey carried out in the Kogi East Senatorial District revealed that cowpea (*Vigna unguiculata*), locally known as "Egwa", is one of the widely acclaimed cultivated grains in the area, and it is highly susceptible to fungal root rot (Agricultural Development Project (ADP) Personal Communication). The economic losses in Nigeria due to fungal attack were equally stressed by Williams (1975).

Fungicides have been used for a long time to curb the detrimental effects of fungi on plants. The systemic fungicide benlate (active ingredient 50% benomyl) was first introduced in 1967 (Wiswesser, 1976; Cremlyn, 1980). Its wide – spectrum systemic activity has been recognized.

Edgington *et al* (1971) described the fungicide as highly toxic to Blastosporae fungi. It is said to be particularly useful as a foliar spray and for seed dressing or soil treatment for the control of grey mold, apple scab, canker, storage and root rots, and leaf spot and for major fungal diseases of soft fruits and vegetables (Cremlyn, 1980). The objective of this research therefore is to isolate and identify the fungal pathogen responsible, for the root rot of cowpea in the East Senatorial District of Kogi State, Nigeria. The study is also intended to determine the economic importance due to fungicides application on root rot disease of cowpea and also to investigate the *in vitro* effects of some fungicides on the linear growth; mycelia dry weight, sporangial formation and germination.

MATERIALS AND METHODS

Collection of sample

Soil samples and infected roots were used. Samples of infested soil were collected from the rhizosphere around apparently diseased plants and brought to the laboratory for analysis from the various field locations. While the infected roots from sample locations were carefully uprooted. They were uprooted as soon as symptoms of rot are noticed.

Isolation of Fungi

Two methods of isolation were used: serial dilution and direct isolation methods. Serial dilution method was used in isolating the fungi from soil samples following the method of Adeniyi and Suleiman (2001) modified by Amoo *et al* (2007). Ten (10) g of the rhizosphere composite soil sample was weighed using a weighing balance and added to 90ml sterile distilled water in a conical flask. This was thoroughly shaken by means of a mechanical shaker for 30 minutes. One (1) ml of the mixture was added to 9ml sterile distilled water in a test tube (now containing 10 ml) and from this mixture another 1ml was taken and added to another tube containing 9ml sterile distilled water. The dilution rates obtained in this form were 10^{-1}, 10^{-2}, 10^{-3}, and 10^{-4}. The same procedure was repeated for the composite samples from each of the three other locations of the study areas. One ml of the soil suspension was taken from each of the test – tubes at different dilution rates using syringe and needle and was inoculated on the agar. For direct isolation from infected roots, several sections (5 – 10 mm) were cut from the margin of the infected lesion to contain diseased and healthy – looking tissue following the methods of Agrios (2005). They were surface – sterilized with 70% alcohol, rinsed in several changes of distilled water and then blotted dry on clean sterile filter paper. These were inoculated on Potato Dextrose Agar (PDA) in 4.5 cm Petri-dishes using sterile forceps. The plates were incubated at 27±2°C and observed at regular intervals. The number of colonies on each plate was counted and recorded.

Determining Effects of (Benlate, Ridomil and Mancozeb) on Mycelial Growth of the Isolate.

The effects of these fungicides in the control of this pathogen were carried out *in vitro*. The weight of each fungicide was calculated to give definite concentrations in parts per million (ppm) of its active ingredient. Stock solutions or suspensions were prepared by adding the desired grammes aseptically to the appropriate ml of sterile distilled water in conical flasks according to Fernando and Linderman (1994). The concentrations used were 50, 100, 150, and 200 ppm, and these were employed for *in vitro* test as amendments on potato – dextrose agar (PDA). After the PDA was autoclaved and cooled to 45°C, one ml of fungicide solution was added to each of the

replicated plates using a graduated sterile syringe apparatus. Then, 5 mm - diameter plugs were cut from actively growing colony margins and placed in the centre of the fungicide – amended medium in three replicate plates per treatment. The cultures were incubated at 27± 2°C in the light/ dark regime. Mycelial growth was measured daily for 6 days. Growth was determined as the mean of two colony diameters taken at right angles to each other, minus the diameter of the inoculum as suggested by Kuthubutheen and Pugh (1978). The resulting figure was divided by two to obtain average radial growth. Mycelial growth (colony diameter) was measured daily. The experiment was a Completely Randomized Design with three replicates, and was repeated. As soon as the control plates were filled up, the results were collated and analyzed. The average radial measurements of the plates were taken. Percentage inhibitions of each of the fungicides at different concentrations were calculated using the formula:

$$\% \text{ Inhibition} = \frac{Diam.\ control\ plates - Diam.\ in\ treated\ plates}{Diam.\ control\ plates} \ x \ \frac{100}{1}$$

Or $\%\ I = \frac{C_0 - C_T}{C_0} \ X \ \frac{100}{1}$ (*Taiga et al*, 2008), where C_o is the diameter in control plates and Ct is the diameter in treated plates.

Effects of the Fungicides on Mycelial Dry Weight of the Isolate

Similarly, as described above, the various concentrations of Benlate and Mancozeb were used. A 5 mm mycelia disk from a 7 – day – old culture was inoculated in each flask such that the mycelium matt was uppermost and floated on the medium. Again each treatment was replicated three times. The cultures were incubated at 27°C ±2°C on the laboratory bench, with occasional gentle hand shaking. Harvesting was carried out at five-day intervals and oven – dried to constant weight on Whatman's filter paper.

Effects of Fungicides on Sporangial Production and Germination of the Isolate.

Evaluation of sporangial production was carried out both on solid (PDA) and liquid media at50,100,150 and 200 ppm of Benlate and Mancozeb. The culture of *Pythium* used to evaluate mycelial growth was used here. The plates were observed every other day from the third day for 21 days. The number of mature sporangia produced on PDA impregnated with various fungicide concentrations was counted for each treatment and replication under the microscope. The number of sporangia per treatment was taken as the mean of three replications. On liquid medium, drops were taken from incubated flasks and observed under the microscope. The sporangia were counted at different concentrations of Benlate, Mancozeb and the control. The number of germinating sporangia was determined and percentage germination at each fungicide concentration was calculated using:

$$\% \text{ germination} = \frac{No\ of\ germ.\ Sporangia}{Total\ no.\ of\ sporangia} \ X \ \frac{100}{1}$$

Similarly, number and germinating sporangia on the control plates was examined and percentage inhibition was also calculated using the formula stated above.

Identification of Isolate.

Pure culture of the isolate was examined under the microscope. The isolated fungus was identified according to the descriptions given by Agrios (2005) and Alexopoulus and Mims (1988). Further confirmation of the fungus was carried out at International Institute of Tropical Agriculture, Ibadan, Nigeria.

DATA ANALYSIS

All results obtained were analyzed using Simple Descriptive Statistics such as mean and standard error. ANOVA statistical test carried out at 5% level of significance. **SPSS 15.0** for windows was used for the statistical analysis. Honestly Significant Difference (HSD) was used for inferential statistical analysis while standard error was used for descriptive statistics.

RESULTS

The isolate (morphology).

The isolated fungus from field infected roots, soil and soil-drenched with suspension from the various field locations studied in the east senatorial district of Kogi state, Nigeria was identified as *Pythium aphanidermatum*. The fungus was found to be associated with root rot of cowpea in the study areas. The fungus has coenocytic (aseptate) hyphae with whitish vegetative mycelium that is richly branched, slender, and cylindrical profusely branching, hyaline and rapidly growing mycelium. The mycelium gives rise to terminal, or intercalary sporangia. The sporangia, which are usually produced in vesicles during sexual reproduction, are globose to oval or at times irregular in shape and germinate directly by producing one to several germ tubes.

Inhibitory Effects of Benlate on Mycelial Growth of *P. aphanidermatum* Grown on Potato Dextrose Agar (PDA).

The fungicide was effective in inhibiting the mycelial extension of the pathogen at all concentrations tested. The mycelial extension decreased with increase in fungicide concentrations **(Table 1)**. The total inhibition of Benlate at 150 ppm shows its effectiveness in inhibiting mycelial growth of *Pythium aphanidermatum*. Besides the inhibition of radial mycelial growth in *Pythium aphanidermatum*, Benlate also affected the growth habit of the fungus as it produced a fluffy, slightly lobbed, aerial mycelium. The inhibitory effects of the fungicide showed level of significance at 0.05% at all levels of concentration compared with the control. The result between 50 ppm and 100 ppm showed no significant difference ($P\ 0.24 > 0.05$). Similarly, between 100 ppm, 150 ppm and 200 ppm was not significant ($P\ 0.31 > 0.05$). Benlate completely inhibited mycelial growth throughout the period of observation at 150 ppm and 200 ppm with no significant difference between them ($P\ 1.00 > 0.05$).

Table 1: Inhibitory Effects of Benlate on Mycelial Growth of *P. aphanidermatum*

Concentration (ppm)	Mean percentage inhibition ± SE (%)
0	0.00 ± 0.0 [c]
50	98.3 ± 0.6 [b]
100	99.2 ± 0.3 [b a]
150	100.0 ± 0.0 [a]
200	100.0 ± 0.0 [a]

Means followed by the same letters are not significantly different ($P \leq 0.05$).

Inhibitory Effects of Ridomil on the Mycelial Growth of *P. aphanidermatum* Grown on Potato Dextrose Agar (PDA).

The fungicide inhibited the mycelial growth at all concentrations tested, but at 40 ppm a scanty and uneven growth was noticed during the preliminary trial **(Table 2)**. No growth at 50 ppm, 100 ppm, 150 ppm and 200 ppm; and these were significantly different compared to the control.

Inhibitory Effects of Mancozeb on the Mycelial Growth of *P. aphanidermatum* grown on Potato Dextrose Agar (PDA).

Mancozeb was found to be effective against mycelial growth of *Pythium*. The effectiveness increased with increase in concentration. There was no significant difference between 50 ppm, 100 ppm and 150 ppm ($P\ 0.7 > 0.05$). Complete inhibition was however observed at 200 ppm **(Table 3)**. Mancozeb significantly reduced mycelial growth at 100 ppm and 150 ppm; with complete inhibition at 200 ppm but the difference was not significant ($P\ 0.84 > 0.05$) between 100 ppm and 150 ppm when compared. Difference between 100 and 200 ppm was equally not significant.

Table 2: Inhibitory Effects of Ridomil on the Mycelial Growth of *P. aphanidermatum*

Concentration (ppm)	Mean percentage inhibition ±SE (%)
0	0.00 ± 0.0 [b]
50	100.0 ± 0.0 [a]
100	100.0 ± 0.0 [a]
150	100.0 ± 0.0 [a]
200	100.0 ± 0.0 [a]

Means followed by the same letters are not significantly different ($P \leq 0.05$).

Table 3: Inhibitory Effects of Mancozeb on Mycelial Growth *P. aphanidermatum*

Concentration (ppm)	Mean percentage inhibition ± SE (%)
0	0.00 ± 0.0^{c}
50	98.8 ± 0.4^{b}
100	99.2 ± 0.3^{ba}
150	99.6 ± 0.2^{ba}
200	100.0 ± 0.0^{a}

Means followed by the same letters are not significantly different ($P \leq 0.05$).

Inhibitory Effects of Benlate and Mancozeb on Sporangial Production in *P. aphanidermatum*.

Sporangia production by *Pythium aphanidermatum* was inhibited by Benlate at 150 ppm and Mancozeb at 200 ppm. With P 0.93 > 0.05, there was no significant difference at 150 ppm and 200 ppm for Benlate. The mean difference of sporangial production was, however, highly significant at all concentrations of Benlate compared with the control at 5% level. For Mancozeb, there was a significant difference at all the concentrations when compared with the control (P 0.00 < 0.05). The fungicides, however, delayed sporangial formation compared with the untreated (control) **(Tables 4 and 5)**. Although the number of sporangial produced in medium containing either Benlate or Mancozeb tended to decrease with rising fungicide concentration, there was no remarkable difference between the two fungicides with respect to sporangial formation. Benlate, however, had more inhibitory effect than Mancozeb.

Table 4: Inhibitory Effects of Benlate on Sporangial Production of *P. aphanidermatum*

Concentration (ppm)	Mean Sporangia No ± SE ($\times 10^4$)
0	39.67 ± 0.3^{d}
50	19.33 ± 0.3^{c}
100	14.33 ± 0.3^{b}
150	0.31 ± 0.3^{a}
200	0.00 ± 0.0^{a}

Means followed by the same letter are not significantly different ($P \leq 0.05$).

Table 7: Inhibitory Effects of Mancozeb on Sporangial Germination of *P. aphanidermatum*

Concentration (ppm)	Mean Sporangia germination ± SE ($\times 10^4$)
0	13.67 ± 0.3^{c}
50	5.67 ± 0.3^{b}
100	5.00 ± 0.0^{b}
150	0.00 ± 0.0^{a}
200	0.00 ± 0.0^{a}

Means followed by the same letters are not significantly different ($P \leq 0.05$).

Effects of Benlate and Mancozeb on the Mycelial Dry Weight of *P. aphanidermatum*.

Benlate and Mancozeb reduced the mycelial dry weight of *Pythium aphanidermatum*. Although Benlate tended to be more effective with increasing concentration, there was essentially no difference between performances of the two fungicides at various concentrations **(Tables 8 and 9)**. Benlate reduced mycelia dry weight by about 75% at 50ppm concentration. The mean difference between the concentrations is significant at 5% level. But there was no significant difference at 150 ppm and 200 ppm (P 0.36 > 0.05). Mancozeb reduced mycelial dry weight by about 93% at the same concentration, showing the level of effectiveness of Benlate over Mancozeb on the mycelia dry weight. There was no significant difference at 50 ppm concentration (P 0.54 > 0.05) compared with the control in Mancozeb. And comparing 100 ppm and 150 ppm (P 0.20 > 0.05) showed no significant difference. Percentages of mycelia dry weight in both fungicides decreased with increase in concentration with the least of 4% in Benlate at 200 ppm and about 3% in Mancozeb at the same concentration (200 ppm) respectively.

Table 8: The Mean Mycelial Dry Weight Growth of *P. aphanidermatum* on Benlate

Concentration (ppm)	Mean dry weight ± SE (g)
0	0.16 ± 0.0^{d}
50	0.12 ± 0.0^{c}
100	0.10 ± 0.0^{b}
150	0.05 ± 0.0^{a}
200	0.04 ± 0.0^{a}

Means followed by the same letters are not significantly different ($P \leq 0.05$).

Table 9: The Mean Mycelial Dry Weight Growth *P. aphanidermatum* on Mancozeb

Concentration (ppm)	Mean dry weight ± SE (g)
0	0.15 ± 0.0^{c}
50	0.14 ± 0.0^{c}
100	0.11 ± 0.0^{b}
150	0.10 ± 0.0^{b}
200	0.03 ± 0.0^{a}

Means followed by the same letters are not significantly different ($P \leq 0.05$).

DISCUSSION

The isolated fungus from field infected roots, soil and soil-drenched with suspension from various field locations of the three cowpea cultivars grown in the four locations studied was identified as *Pythium aphanidermatum* (Edson) Fitzp.). *Pythium aphanidermatum* is thus implicated in this study as the causal organism of root rot of cowpea disease in the locality. This agrees with the report of Williams (1975). The investigation which was undertaken at four different locations revealed that root rot disease of cowpea is prevalent in the East Senatorial District of Kogi State, Nigeria. The study provided information serving as a base line in establishing *Pythium aphanidermatum* as the causal organism of root rots of cowpea (*Vigna unguiculata* (L.) walp) in the study areas. *Pythium aphanidermatum* was reported by Williams (1975), as a major fungal disease of cowpea in Nigeria. According to him, it is widely distributed where cowpea are extensively grown. This has been confirmed by the present findings in the four locations sampled that *Pythium* is the major agent of root rot of cowpea. The fungus is also known to cause damping off disease of seedlings and affect plants by attacking and killing root tips of plants which are important in taking up nutrients and water, thereby leading to the plant being stunted, wilted and dying off (Dutta, 2005). The pathogen responsible for the rot probably enters the host through wounds inflicted by soil nematodes apart from germ tube that can pearse and penetrate their hosts (Dutta, 2005).

The isolated fungus associated with root rot of cowpea which was identified as *Pythium aphanidermatum* had all the morphological features of *Pythium* described by Kelmsdal *et al.* (2007) and Agrios (2005). The presence of coenocytic hyphae provides evidence of *Pythium,* with globose to oval or an irregular shaped sporangia. The fungus has coenocytic hyphae with whitish vegetative mycelium that is highly branched, slender, and cylindrical, hyaline and fast growing mycelium. The mycelium gives rise to terminal, or intercalary sporangia. The sporangia, which are usually produced in vesicles during sexual reproduction, are globose to oval or at times irregular in shape and germinate directly by producing one to several germ tubes. In addition to non-septate hyphae, the sporangia produce vesicle during sexual reproduction.

Generally, fungicides have for a long time been used against pathogenic micro organisms to help curb their detrimental effects on plants and animals which directly or indirectly affect the well

being of human beings. Of the three fungicides tested *in vitro*, Ridomil was most effective at low concentrations (40 ppm) in inhibiting mycelia growth of *Pythium aphanidermatum*, a confirmation of Lobna (2006), that root rot of squash can be controlled with Ridomil. The study also confirms low effectiveness of Benlate and Mancozeb unless used at high concentrations.

The present investigation has shown that Benlate and Mancozeb progressively inhibited the radial mycelia growth of *Pythium aphanidermatum* on potato dextrose agar. Benlate (100 ppm) inhibited mycelia growth by 99.2%, while Mancozeb (150 ppm) could only achieve 99% inhibition compared to the control plates. These results seem to differ from those of Wokocha and Ebenebe (1980) who reported that Benlate had little or no effect on mycelia growth of soil fungus when applied at 500 ppm concentration in an *in vitro* experiment. The effectiveness of Ridomil at relatively low concentration suggests that it could be used in combination with other very effective fungicides (Fernando and Linderman, 1994).

Effects of these fungicides on mycelia dry weight on *P. aphanidermatum* showed that the highest mean mycelia dry weight (140 mg) was recorded at 50 ppm in Mancozeb while Benlate had 120 mg at the same concentration. The two fungicides continued to show decline in mycelial dry weight with increase concentrations, this observation agrees with the work of Fernando and Linderman, (1994). The potency of these fungicides has been attributed to its ready dissociation in solution. Their mode of action is said to be on mitotic division, probably by the interference with the spindle (Gillian, 2006). There is good evidence that these fungicides are xylem- and phloem- translocated, very effective as soil drenches in controlling and capable of killing soil fungi (Lobna, 2006). It was observed in the present study that the maximum infection which may have occurred in cowpea plants from planting till flowering was controlled with the applications of these fungicides. Mean percentage infestation of treated samples of 6.7% as against 33.3% in control suggests an economic gain. Therefore, farmers and or researchers are positively disposed to the use of these fungicides in order to sustain the economic value of the crop.

The higher sensitivity of the mycelium of *P. aphanidermatum* to these fungicides may be due to any of the following factors:

(i) Differences in the mode and sites of action and in the degree of solubility of the fungicides in water;

(ii) Differences in rates of absorption of the fungicides by the fungus or possible detoxification of the fungicides by the fungus.

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