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Full Length Research Paper

Media studies on *Myrothecium roridum* Tode: A potential biocontrol agent for water hyacinth

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Water hyacinth (*Eichhornia crassipes*) is a noxious aquatic weed in Nigeria and many parts of the world. A potential mycoherbicidal agent for the control has been identified recently as *Myrothecium roridum* Tode. The best media for *in-vitro* propagation was investigated using seven culture media; potato dextrose agar (PDA), malt extract agar (MEA), potato sucrose agar (PSA), sabouraud agar (SA), potato carrot agar (PCA), Czapek-Dox agar (ZA) and a semi artificial diet, which included the material from the fungal host's plant (WHA). The effect of nitrogen sources (ammonium chloride, sodium nitrate, ammonium nitrate, sodium glutamate and glutamine) and pH on the growth of the fungus was also determined. The mycelia growth was assessed by diameter measurement on agar plates and the conidial yield was measured with a Neubauer hemocytometer slide. The mycelia growth was maximum on PSA and minimum on ZA. The conidial yield was highest on MEA. The mycelia growth and spore concentration of the fungus were highest on sodium glutamate and glutamine respectively, when used as nitrogen sources. The optimal growth pH was 5.5.

Key words: Media, Mycoherbicide, *Myrothecium roridum* Tode, nitrogen sources.

INTRODUCTION

The genus *Myrothecium* is known to contain several species, mostly saprophytic (Quezado, 2010). This organism belongs to the order Hypocreales (Kirk et al., 2008) with an uncertain family position. *Myrothecium roridum* is a soil fungus and survives in this environment as a saprophyte in decaying plant tissues (Souza-Motta et al., 2003; Costa et al., 2006; Domsch et al., 2007).

Despite its saprophytic nature, the organism is beset with several economic importance. *Myrothecium* is able to cause diseases, mainly in the aerial parts of some plant species (Ahrazem et al., 2000; Domsch et al., 2007). It is a facultative parasite with a large number of plant hosts, including vegetables, fruits and ornamental plants. (Tulloch, 1972; Mendes et al., 1998; Poltronieri et al., 2003; Murakami and Shirata, 2005; Silva and Meyer, 2006).

M. roridum has been reported to produce toxins in culture medium. These toxins have been shown to have a potential antibiotic effect against a wide range of clinical and environmental bacteria and fungi such as: *Bacillus subtilis, Clavibacter michiganensis pv. Michiganesis, Botrytis cinerea, Colleotricum acutatum, Colleotricum demantium, Rosellinia necatrix, Sclerotia sclerotiorum, Diaporthe nomurai and Cochliobolus miyabeanus* (Turhan and Grossmann, 1994; Murakami et al., 1998).

Myrothecium species are known to produce a range of cellulolytic enzymes (Moreira et al., 2005; Okunowo et al., 2010). These enzymes have biotechnological use in food and pharmaceutical industries, paper industries, biomass conversion of agricultural and industrial wastes to chemical feedstock, biofuels, animal feeds and pollution control (Murao and Tanaka, 1982; Viikari et al., 1994; Christov et al., 1999; Zaldiva, 2001; Ikram-ul-Haq et al., 2006; Tarek and Nagwa, 2007; Acharya et al., 2008; Okunowo et al., 2010).

Myrothecium roridum and some related species have been reported to produce tumor-inhibiting compounds (macrocyclic trichothecenes), which are active against the growth of cancer cells including lung, hepatic, breast and prostate cancer (Schoettler et al., 2006; Xu et al., 2006; Ge et al., 2009).

The organism, *M. roridum* has been shown to posses

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the potential that should be used as a broad spectrum bioherbicide in the control of water hyacinth and other weeds (Ponappa, 1970; Hettiarachchi, 1983; Liyanage and Gunasekera, 1989; Lee et al., 2008). Also, a potential mycoherbicidal agent for the control of water hyacinth in Nigeria has been identified in a recent study as *Myrothecium roridum* Tode (Okunowo et al., 2008).

Despite the immense biotechnological relevance of the fungus, little information is available on the requirements for growth and sporulation. This research will provide valuable information to further mycological and pathological research on fungus and disease, including development of measures for disease management. The objective of this study was to investigate the effects of culture media and nitrogen sources on mycelial and conidial growth with the view of developing low cost substitute for expensive laboratory media in mass production of the fungus.

MATERIALS AND METHODS

Fungal isolate

The strain of *Myrothecium roridum* Tode was isolated from water hyacinth, characterized and deposited in the Center for Agriculture and Bioscience International, United Kingdom and was given the accession number: IMI394934. A lyophilized sample of the isolate was reconstituted in petri dishes according to Okunowo and Ogunkanmi (2009). This was done by adding 20 ml sterile distilled water into a test tube containing a sample of the lyophilized organism. The test tube was agitated and an aliquot of the cell suspension was obtained with the aid of a sterile syringe. A 2ml suspension was added to a petri dish containing potato dextrose agar and this was incubated for 7 days at 25°C.

Culture media preparation

Seven culture media were used to assess the mycelia and conidial growth of the fungus, *Myrothecium roridum* Tode (IMI394934). The media were: potato carrot agar (PCA) medium (potato 20 g, carrot 20 and agar 20), potato dextrose agar (PDA) medium (diced potato 200 g, dextrose 15 and agar 20), sabouraud agar (SA) medium (glucose 40 g, peptone 10 and agar 15), water hyacinth agar (WHA) medium (dried powdered water hyacinth leaf 50 g, agar 18 and fresh hyacinth leaf extract {100 g/liter of distilled water}) and Czapec-Dox agar (ZA) medium (sodium nitrate 2 g, potassium nitrate 1, potassium chloride 0.5, magnesium sulphate 0.5, ferrous sulphate 0.01, sucrose 30 and agar 20). The media also included malt extract agar (MEA) medium (malt extract 20 g and agar 20) and potato sucrose agar (PSA) medium (potato 200 g, sucrose 20 and agar 20). Each culture medium was prepared in a liter of distilled water and autoclaved at 120°C at 15 psi for 20 min (Okunowo and Ogunkanmi, 2009).

Assessment of the media effect on mycelial growth of *M. roridum*

The mycelial growth of the organism was determined by the measurement of the isolate's colony size (Okunowo and Ogunkanmi, 2009). Two bisecting lines were drawn on the lower part of a sterile Petri dish. Each prepared medium (10 ml) was added into different sterile Petri dishes and was allowed to solidify. A mycelia plug of the isolate was cut with a flame sterilized 8 mm diameter cork borer and placed in the centre of each of the Petri dishes at the point of bisection. These were placed in an incubator (Gallenkamp SG91/08 /717, Loughborough, UK) at 25°C. The growth diameter (colony size) of the isolate was measured along the two bisecting lines on a daily basis and the average diameter measurement was recorded in millimeter. Each growth medium was replicated on 5 plates.

Assessment of the media effect on conidial growth of *M. roridum*

To determine the influence of media on conidial growth, the organism was cultivated in media types as described above for a period of 14 days. The conidia (spores) of the organism were harvested from each culture plate and the concentration was estimated for each medium using a Neubauer hemocytometer slide. This was done by adding 1 ml of sterile distilled water (containing 0.1% v/v Tween 80 solution) into the Petri dish. The spore suspension obtained was diluted as appropriate. A drop of the suspension was made into a Neubauer hemocytometer slide and the spores were estimated as:

$$\text{Spore concentration (Spores/ml)} = \frac{\text{Number of spores counted X Dilution factor X } 10^6}{\text{Number of area counted}}$$

The spore concentration was recorded as mean value from five replicated plates.

Assessment of nitrogen sources' effect on mycelial growth of *M. roridum*

To determine the effect of nitrogen sources on mycelial growth of the organism, Czapek-Dox agar medium (sodium nitrate 2 g, potassium nitrate 1, potassium chloride 0.5, magnesium sulphate 0.5, ferrous sulphate 0.01, sucrose 30 and agar 20) was prepared such that it contains one of the following as nitrogen source (2 g/litre): ammonium chloride, ammonium nitrate, glutamine, sodium glutamate and sodium nitrate. These were also autoclaved, inoculated and used for colony size measurement as above.

Assessment of nitrogen sources' effect on conidial growth of *M. roridum*

The conidia of *M. roridum* were harvested from the culture plates containing the different nitrogen sources on day fourteen (mean time taken for maximum mycelial growth in the media used) and the Neubauer count was estimated as described previously.

Assessment of pH effect on growth of *M. roridum*

The effect of pH on the growth of *M. roridum* was investigated as described by Okunowo et al. (2009). The fungus, *M. roridum* was grown on potato dextrose agar plates for 14 days.

Growth media with a range of pH was prepared as follows using buffer solutions prepared by mixing different volumes of both sodium and potassium dihydrogen phosphate solution (Table 1). A 400 ml potato dextrose broth medium (PDB) was also prepared. The broth was divided into five equal portions of 80 ml labeled A to E. The pH of A was adjusted to 4.6 with a pH meter (Cole-Parmer 60648, Chicago, USA) and 20 ml of the buffer of the same pH

Table 1. Preparation of buffer solution for microbial growth.

Vol. of Na_2HPO_4 stock (11.88 g/litre) (ml)	Vol. of KH_2PO_4 stocks (9.08 g/litre) (ml)	*Approx. pH of buffer
0	20	4.6
2	18	5.8
12	8	6.7
19	1	7.8
20	0	9.0

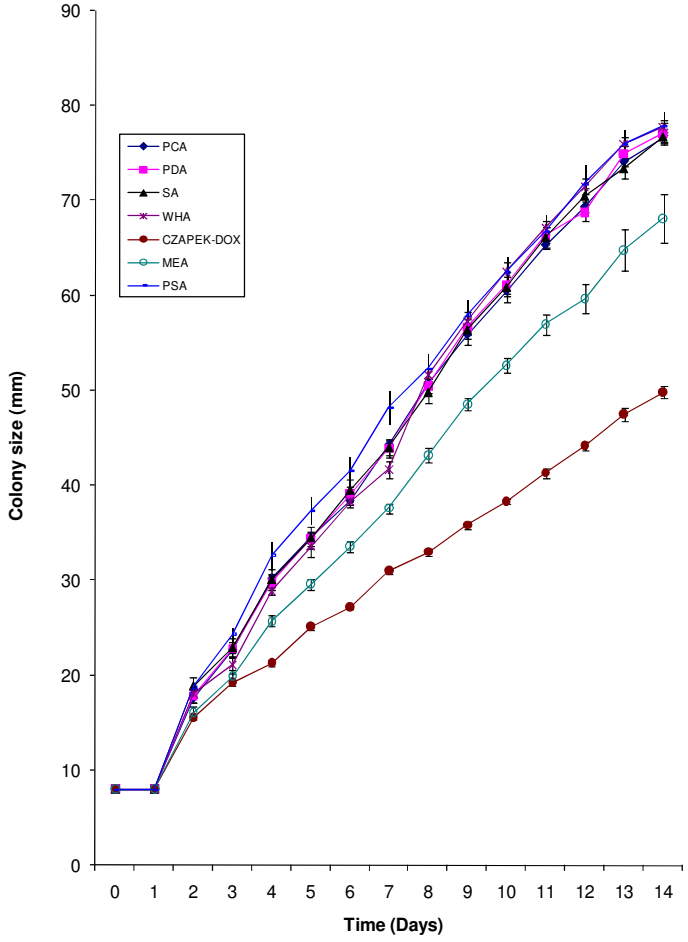

Figure 1. Growth profile of *Myrothecium roridum* Tode (Culture IMI 394934) on media types. Values are mean ± SEM of five replicate results.

(Table 1) was added. The mixture was distributed (25 ml) into three 100 ml flasks and were labeled A. The pH of the medium lot B was adjusted to 5.8, C to 6.7, D to 7.8 and E to 9.0. The same procedure was carried out on B, C, D and E as in A. All 15 flasks were autoclaved at a temperature of 120°C at 15 psi for 20 min.

Spore suspension of the fungus was produced by adding 10 ml sterile distilled water (SDW) containing 0.1% (v/v) Tween 80 to two of the 14 days old PDA culture plates of *M. roridum*. These were gently agitated and the spore suspensions were aseptically pooled together in a sterile 100 ml flask. With the aid of a sterile pipette, 1 ml of this spore suspension was made each into the 15 flasks A to E. These were allowed to stand on a shaker at laboratory condition

Table 2. Average growth rate (mm/day) of isolates on media types.

Media types	Average growth rate (mm/day)
PCA	4.69 ± 0.13*
PDA	4.82 ± 0.21*
SA	4.40 ± 0.41*
PSA	7.45 ± 1.65
WHA	4.81 ± 0.50*
ZA	2.64 ± 0.12*

[1]Data represent average growth rate ± S.E.M of triplicate results derived as growth rate (μ) = ΔS/ΔT. Where ΔS = Colony size (mm) and ΔT = Time (Days). Medium with highest growth rate was compared to others using the Student's t-test and ANOVA, where *P < 0.05.

for 24 h. The content of each flask was harvested in a centrifuge (Surgifriend Medicals SM902B, England) at 3,500 g for 10 min. The pH of the supernatant was measured. The pellets were resuspended in the same volume of water (25 ml) and the optical density was measured at 530 nm in a spectrophotometer (Thermo Spectronic 4001/4, USA).

Data analysis

Data were given as mean ± SEM of measurements from 5 plates replicate containing the same medium, except otherwise stated. Statistical significant difference in the rate of microbial growth on media types was assessed by a one-way analysis of variance with probabilities of less than 0.05 considered significant. Statistical analysis was done using a computer software programme (Microsoft Excel, 2003).

RESULTS

Effect of media on the growth of *M. roridum*

All the media supported the growth of the fungus to various degrees (Figure 1). The mycelia growth on day 14 was maximum on PSA and minimum on ZA (Figure 1). The growth rate of the organism was determined on all the media types as colony size per day. The result obtained showed that the organism had a different growth rate on each medium (Table 2). The highest growth rate was recorded on PSA and this was significantly greater

Table 3. Neubaura count of *M. roridum* on media types.

Media type	Conidial yield (Spore / ml) x 10^7
ZA	0.160 ± 0.00*
TWA	1.00 ± 0.00*
WHA	3.90 ± 0.5*
PCA	3.98 ± 0.01*
SA	5.37 ± 0.08*
PDA	26.4 ± 4.77*
PSA	322.00 ± 8.52*
MEA	2990.00 ± 7.89

Values are mean ± SEM of five replicate results. Medium with highest growth rate was compared to others using the Student's t-test and ANOVA, where *P < 0.001.

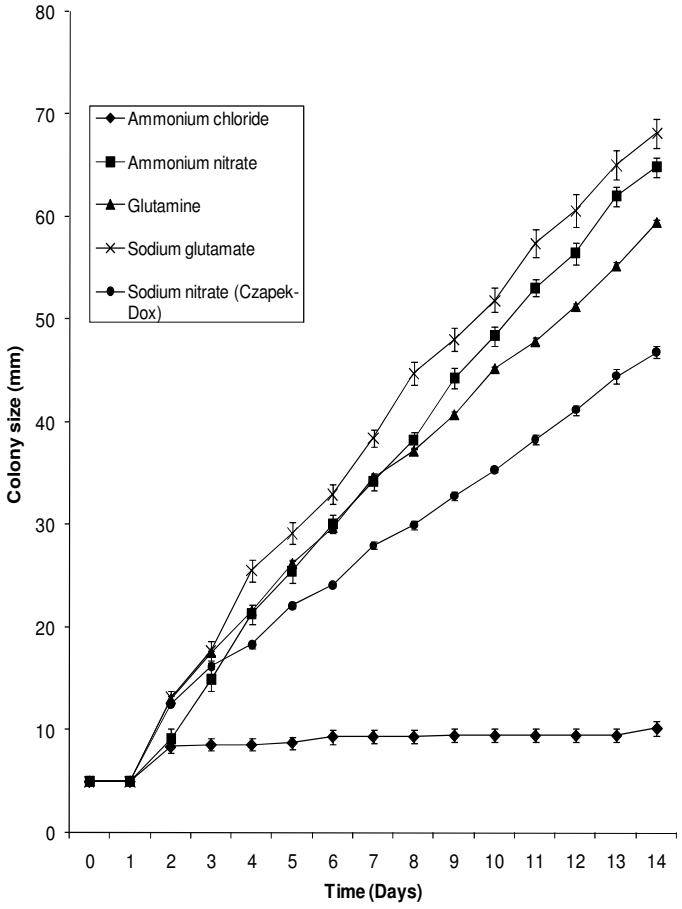

Figure 2. Growth profile of *Myrothecium roridum*Tode (Culture IMI 394934) on nitrogen sources. Values are mean ± SEM of five replicate results.

than those obtained on other media types such as MEA and Czapek-Dox (P<0.05). It should also be noted that water hyacinth leaf agar (WHA) also supported the mycelia growth of the organism and this was the cheapest medium used in this study. This also suggests that the mass production of the organism using the formulated water hyacinth leaf agar (WHA) medium may be economical. Sporulation of *M. roridum* was best on MEA, followed by PSA and the least was observed on ZA (Table 3). The spore concentration of this isolate on MEA was significantly greater than those of other media types used (P<0.001).

Effect of nitrogen sources on the growth of *M. roridum*

The fungus was also grown on basal salt medium. Its nitrogen salt was substituted with different nitrogen sources and the mycelial and conidial growth was monitored. The result obtained showed that the mycelia growth of the fungus was maximum on sodium glutamate and least on ammonium chloride containing medium, respectively (Figure 2). More so, the highest amount of spores was recorded on glutamine containing medium (Table 4).

Effect of pH on the growth of *M. roridum*

The influence of pH on fungal growth was determined by turbidometry measurement of the fungal spores in aqueous solution. The result obtained showed that the fungus was able to grow over the pH range (5.5 to 8.6) employed in this study. The growth of the organism was highest at pH 5.5 and lowest at 8.6 (Figure 3).

DISCUSSION

Effect of media on the growth of *Myrothecium roridum* Tode

In this study, the mycelia growth of *M. roridum* was most favored on potato sucrose agar (PSA), water hyacinth agar (WHA) and potato dextrose agar (PDA), although, PSA and PDA are commonly used in the isolation and propagation of the fungus (Hettiarachchi et al., 1983; Gees and Coffey, 1989; Murakami et al., 1998; Kim et al., 2003; Magandi et al., 2007; Lee et al., 2008; Duval, 2010). WHA competed favourably with both PSA and PDA in terms of mycelia growth support for the organism. Many phytopathogenic organisms have been found to thrive well on medium containing the host plant extract (Peters et al., 1998; Zimpfer et al., 2004; Kuwada et al., 2006; Okunowo and Ogunkanmi, 2009). This may be due to the nutrient composition of the host material. The medium, WHA, is known to be a rich source of minerals, proteins, sugars and lipids which may be utilized by the organism for metabolic activities. The proximate composition of water hyacinth leaves have been previously reported (Igbinosun et al., 1988; Okunowo and Ogunkanmi, 2009).

Table 4. Neubaura count of *M. roridum* on nitrogen sources.

Nitrogen sources	Conidial yield (spore /ml) x 10^6
Ammonium chloride	0.30 ± 0.00*
Sodium nitrate (Czapek Dox)	1.85 ± 0.07*
Ammonium nitrate	2.00 ± 0.01*
Sodium glutamate	74.50 ± 0.58*
Glutamine	91.10 ± 2.86

Values are mean ± SEM of five replicate results.
Medium with highest growth rate was compared to others using the Student's t-test and ANOVA, where *P < 0.05.

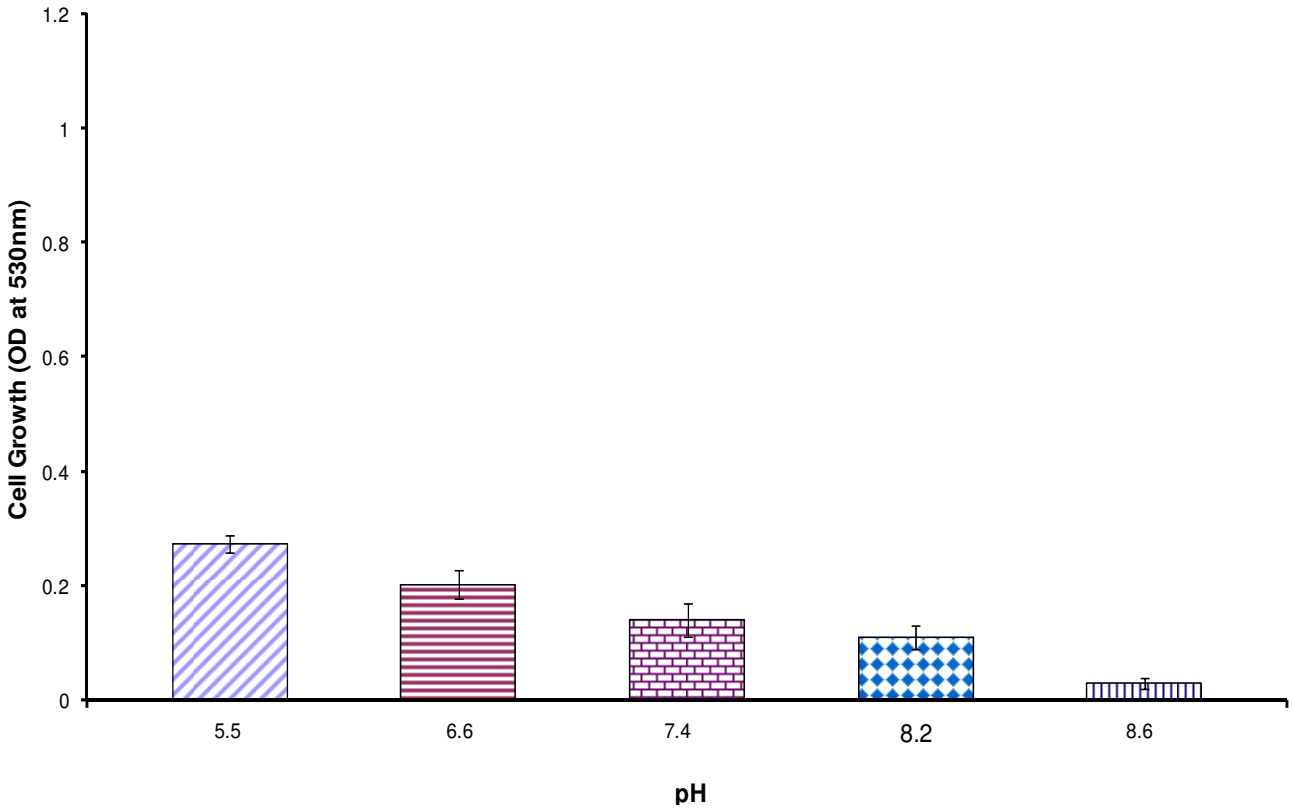

Figure 3. Optimal growth pH for *Myrothecium roridum* Tode (IMI 394934). Values are mean ± SEM for triplicate results.

In addition, studies have shown that the presence of flavonoids in the host plant containing growth formulations stimulates mycelia growth in some plant pathogenic organisms (Chabot et al., 1992; Kuwada et al., 2006).

The medium, WHA has been reported in a previous study as a cost effective medium in propagating *C. pallescens* Boedjin, an isolate from water hyacinth (Okunowo and Ogunkanmi, 2009). This suggests that the medium WHA may be considered for use in mass propagation of the organism as against the use of other expensive media.

Effect of nitrogen sources on the growth of *Myrothecium roridum* Tode

The mycelia growth of *M. roridum* was least favored on Czapek-Dox agar medium. This medium has been reported as a poor medium for the growth of fungi (Nwodo, 2007). This may be due to an inhibition in the growth of the fungus as a result of the chlorine present in the form of potassium chloride in the Czapek-Dox agar medium. Chlorine inhibits the growth of microorganisms and therefore, it is used in the formulation of some disinfectants and germicides (Davis et al., 1994; Gottardi

and Nagl, 2005; Fukuzaki, 2006; Fawley et al., 2007; Kim et al., 2008; Zhu et al., 2008; Bengtson et al., 2009).

In addition, the organism sporulated significantly ($p < 0.01$) on malt extract agar medium when compared to other media. This medium is one of the most widely used media to induce sporulation in fungi (Bills and Polishook, 1992; Guo et al., 1998; Payne, 2009). It is a rich medium due to the presence of malt extract which is often considered as a good complex organic nitrogen source for fungal metabolism (Adejoye et al., 2006; Gbolagade, 2006). The fungus, *M. roridum* sporulated least on Czapek-Dox agar medium and this may also be due to the presence of chloride ion in the medium.

The mycelia growth of the organism on different nitrogen sources was found to be highest on sodium glutamate containing medium and lowest on ammonium chloride containing medium. This result is similar to that obtained in a previous growth study on *C. pallescens* Boedji (Okunowo and Ogunkanmi, 2009) and it suggests that most organic nitrogen salts are easily metabolized and are preferred to inorganic nitrogen salts for fungal growth (Stott and Bullerman, 1975; Adejoye et al., 2006; Gbolagade, 2006). Sodium glutamate and glutamine were the two organic nitrogen sources used in this study, but the mycelia growth was more favored on metal ion-containing organic nitrogen, which is an indication that sodium ion is needed for the metabolic activities in *M. roridum*. Also, sodium glutamate and nitrate were the two nitrogen sources which contained a sodium ion, but the mycelia growth of the fungus was more favored on sodium glutamate. This suggests that an organic nitrogen source containing sodium metal is more preferable for the mycelia growth of the fungus.

However, the highest conidial growth was recorded on glutamine containing medium, followed by sodium glutamate containing medium, which is an indication that sodium metal may not be necessary for conidial growth.

The fungus grew over a wide pH range, but the optimal growth pH was 5.5, which is an indication that this strain of organism may be acidophilic in nature. Therefore, it needs a slightly acidic medium for maximum metabolic activities such as: nutrient assimilation, enzymatic hydrolyses and energy generation in the organism leading to an increased fungal growth. Myrothecium species grows at a wide pH range between 3.5 and 9.5, but the optimal growth pH range from 5.0 to 7.0 (Murao and Tanaka, 1982) agrees with the result obtained in this study.

Further studies will include the investigation of the host range of this isolate and the effect of growth media and nitrogen sources on its virulence on water hyacinth.

Conclusion

In this study, a cost effective medium (containing water hyacinth leaf extract) for the propagation of *M. roridum* has been formulated. This medium was shown to compete favourably with other conventional and expensive media in terms of mycelia growth support for this organism.

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