BIOCONTROL EFFICACY OF A NEW STRAIN OF *MYROTHECIUM RORIDUM* ON *EICHHORNIA CRASSIPES* (WATER HYACINTH) IN LAGOS STATE NIGERIA

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(Matric No.: 209091026)

2008

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A THESIS SUBMITTED TO THE SCHOOL OF POSTGRADUATE STUDIES, UNIVERSITY OF LAGOS IN FULFILMENT OF THE REQUIREMENTS FOR THE AWARD OF DOCTOR OF PHILOSOPHY (Ph.D) IN BIOCHEMISTRY.

UNIVERSITY OF LAGOS
SCHOOL OF POSTGRADUATE STUDIES

CERTIFICATION

This is to certify that this thesis:

Biocontrol Efficacy of a New Strain of *Myrothecium roridum* on *Eichhornia crassipes* (Water Hyacinth) in Lagos State Nigeria

Submitted to the School of Postgraduate Studies, University of Lagos

For the award of the degree of

DOCTOR OF PHILOSOPHY (PhD)

is a record of original research work carried out

By

OKUNOWO WAHAB OLUWANISOLA

In the Department of Biochemistry

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DEDICATION

I dedicate this work to GOD almighty for the journey so far and the beginning of a new dawn.

ACKNOWLEDGEMENTS

This research work was carried out successfully with the concerted efforts and contributions from brilliant minds within and outside the University of Lagos and within and outside the shore of the country. Firstly, I wish to acknowledge the immeasurable support of my supervisors; Professor G.O. Gbenle who initiated the project and financed an aspect of the work, Dr A.A. Osuntoki for being there as my big brother with his radical and constructive criticism and Dr A.A. Adekunle who identified some of the fungal species and the plants used and also contributed some lofty ideas to the work. I wish to appreciate the effort a seasoned Professor of Pharmacy (Professor Olugbade, H.O.D. Central laboratory, Obafemi Awolowo University) who gave me some hands on experience on thin layer chromatography in order to isolate the active component of the crude phytotoxin. I also wish to acknowledge the support of Dr. (Mrs.) Owolabi Mbang of the Department of Pharmaceutical Chemistry for providing some analytical grade precoated silical gel TLC plates which were used for the fractionation of the crude phytotoxin. I wish to thank Professor (Mrs.) O.A. Magbagbeola for all her encouragements and suggestions in the time of adversity on the project. I also wish to thank my brother Okafor Uzoma (Chairman) for supporting me with all his resources be it intellectual, physical, spiritual and financial in order that this work can come to a pass. I sincerely acknowledge the support of my senior colleagues; Prof. A.I. Akinwande, Dr. O.A.T. Ebuehi and Dr. (Mrs.) Odesanmi for beautifying the work with their contributions at seminars. I give thanks to Mrs Solabi (Chief Technologist) for giving me the opportunity to use the lyophilizer and pass nights in her office during the course of lyophilizing the crude toxin. I wish to acknowledge the effort of my bother; Okunowo

Lukman for assisting me in the various aspects of the field work. I thank my fianceé Dr. (Miss.) Ayeteru Mutiat for her patience with me and perseverance in making sure that this project is completed. I sincerely appreciate the various contributions from my colleagues; Mrs. Ezikpe, Mrs. M.N. Imaga, Mrs. Bakare, Mrs. Samuel, Mr. Akande, Mrs. Bolawa and the technical staffs; Mr Ojomo, Mr Apata, Mrs Olusola, Mr Sola, Sister Bisi and Mr Adenekan. I also thank my brothers Olugbenga Okunowo and Jelil Okunowo for their concern. Indeed I have realized that Ph.D work is spiritually, financially, intellectually, politically and physically demanding and these are a collective responsibility of a group of brilliant minds. Therefore, I wish to thank these people once more for collectively contributing their own quota through this research work to humanity and national development.

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ABSTRACT

The use of chemical herbicide in the management of aquatic weeds does not only constitute environmental and health problem but its cost and its non-specific nature calls for the use of biological control agents. An investigation was conducted into the probable use of indigenous fungi as potential mycoherbicides for water hyacinth (*Eichhornia crassipes*) control. Trips were made to Badagry Creeks, Mile 2 and Lagos Lagoon in Lagos State and Ogun river in Isheri, Ogun state to collect samples of diseased water hyacinth plants. Phytopathogenicity of the organisms from the diseased plant were carried out on both water hyacinth plant and some crops. The best medium, nitrogen source and the optimal pH for the growth of the fungi were also determined. The cellulolytic enzymes activities and phytotoxin production of the fungal isolates were also investigated. Six different fungal species (*Aspegillus niger, Aspergillus flavus, Penicillium* sp., *Curvularia pallescens, Fusarium solani* and *Myrothecium roridum*) were isolated from the diseased plant. No disease symptom was observed on the inoculated crops and water hyacinth plant with all the isolates except a strain of *Myrothecium roridum* Tode which showed a characteristic symptom on *E. crassipes*. Necrosis was observed on the healthy plant 3 days post inoculation with $1x10^6$ spores/ml of *Myrothecium roridum*. The leaves and the petioles were observed dead at the end of the third week post inoculation. The Disease Incidence (DI) and the Mean Disease Severity (MDS) was 100% and 8.67 ± 0.33 respectively, on day 24 post inoculation. The best and cheapest growth medium for *C. pallescens* Boedjin and *F. solani* was Water Hyacinth Formulated Agar Medium and Tap water agar for *Myrothecium roridum* Tode (IMI 394934). The growth of *C. pallescence* and *Myrothecium roridum* Tode (IMI 394934)

were highest on sodium glutamate and that of *F. solani* was highest on sodium nitrate when used as nitrogen source in the enrichment medium. The optimal growth pH was 5.5 for *C. pallescens* Boedjin and *Myrothecium roridum* Tode (IMI 394934) and 6.6 for *F. solani*. The cellulose enzyme production was highest in *C. pallescens* Boedjin and lowest in *Myrothecium roridum* Tode when carboxymethylcellulose, sawdust and water hyacinth leaf were used as carbon sources. Of all the isolates only *Myrothecium roridum* was able to produce phytotoxin, which is toxigenic on water hyacinth and four different types of crops. The phytotoxin production by this isolate was dependent on photoperiod, pH, media type, carbon source and nitrogen source. The phytotoxin was thermostable and the biological activity was pH dependent and independent of photoperiod. The purified toxin was colourless in solution, non UV reactive but detected on reaction with vanillin sulphuric acid. Molecular analysis of the ITS rDNA of this isolate showed >98% homology to authenticated sequences of *M. roridum*, and 99% homology to a strain identified as the closely related species *M. carmichaelii*. In this study, the efficacy of the isolates was dependent on their ability to produce phytotoxin. The phytopathogenic isolate, *Myrothecium roridum* possesses the level of virulence needed to be an effective bioherbicidal agent. Hence; it was considered to be a suitable mycoherbicide for use in the management of water hyacinth in Nigeria.

CHAPTER ONE

1.0 INTRODUCTION

During the last 100 years, water hyacinth *Eichhornia crassipes* (Marts.) Solms-Laubach, has been spread by man from its native range in Central and South America to the tropical and subtropical regions throughout the world. This attractive plant spread from natural lakes, watercourses and man-made impoundments and the resulting infestation has caused greater problems than those of any other floating aquatic weed, particularly in North America, Africa, India, South-east Asia and Australia (Harley, 1988). Water hyacinth was first introduced into Africa via Egypt between 1879 and 1892 (Harley, 1988). It made its entry into Nigerian waters via the Southwestern coastal border of Badagry around 1984 (Oso, 1988).

Water hyacinth is the most pernicious weed in the world (Bateman, 2001). It forms dense impenetrable mats that impede the recreational use of water (tourism), irrigated agriculture (infestation of channels) and power generation (loss of electricity production). These mats competitively exclude native submerged and floating-leaved plants and create good breeding conditions for mosquitoes and other disease vectors (Forno and Wright, 1981).

Coupled with the environmental pollution resulting from oil exploration activities and natural phenomena such as perennial flooding and coastal erosion in some countries, these invasive plants do not only block boat traffic (navigation), which is the only means of reaching most parts of the states but also disrupt fishing operation (reduction in fish catch), the main occupation of the riverine people. These losses caused by the weed in the several key sectors of the African countries have been estimated to be in the order of

billions of dollars annually (Mailu, 2001). The indirect economic effects and social costs cannot be overestimated.

Series of control measures such as manual, mechanical, chemical and biological control have been employed in the time past to check this aquatic weed. Some of these methods are expensive and not environmentally friendly.

Manual removal of aquatic weeds is suitable only for very small areas. It is also difficult, labour intensive and could involve serious health risks associated with the work, since the weed provides breeding ground for mosquito, snakes and bilharzias (Mailu, 2001). Transportation of harvested water hyacinth, for example, is also costly because it has very high water content. However, this method induces capacity building through involvement of the local people, and could also create income generation opportunities through uses of the harvested weeds.

Mechanical removal of aquatic weeds is generally seen as the best short-term solution to the problems created by the weeds. It is however costly, involving either land-based bucket cranes, draglines or booms, or alternatively, water-based machinery such as mowers, dredges, barges or specially designed aquatic weed harvester. Even then, such methods are suitable only for relatively small areas; and many of these techniques require the support of water and land-vehicles for transporting the large quantities of the weed that is removed. The relatively high costs of the purchase and maintenance of the machinery are likely to outweigh the positive but limited and temporary impact of the method.

The application of herbicides for the control of aquatic weeds has been carried out for many years. For example, the common herbicides for controlling water hyacinth are

glyphosate, diquat and 2,4-D ethyl ester. There is a good success rate when applied on small infestations but less successful with larger areas. Application can be from the land or from the air; of which either way, requires skilled operators. The main concern on the use of herbicides is the environmental and health related effects, especially where people drink from the water. The method does not give a permanent solution to infestation by the weeds.

Biological control is the use of host specific natural enemies to reduce the population density of a pest. Several insects and fungi, including a variety of weevils, moth and fungi have been identified as control agents for aquatic weeds. The bio-agents act strictly specifically on the weeds and remain available at the site of infestation, and tend to be self-regulating. The biological control is therefore said to be environmentally safe. Usually, it takes about 1-2 years for the biological agents to substantially reduce the weeds population in the case of water lettuce or water fern, and 3 years or above in the case of water hyacinth. However, the duration depends on the host and the agent.

Some authors (Shabana *et al.*, 1995b; Bateman, 2001) prefer the use of biological control agent over the use of conventional control measures such as mechanical removal, chemical herbicides (glyphosate and 2, 4-D) and classical biological control, using herbivorous insects. Herbicides have the added disadvantage that they might have adverse environmental effects and must be applied carefully and selectively. They also interfere with or nullify the action of biological control agents present (Charudattan, 1995). Currently, about 15 to 20 weeds are biologically controlled with plant pathogens worldwide (Rosskopf *et al.*, 1999; Evans and Reeder, 2001). Extensive research has been done on the exploitation of different pathogens as mycoherbicides for water hyacinth

control (Shabana, *et al.*, 1995b; Shabana, 1997; Shabana *et al.*, 1997; Charudattan, 1997; Evans, 2000). Some plant pathogens can cause significant reductions in water hyacinth biomass, especially following natural disease outbreaks, after severe insect attacks, or when used as inundative bioherbicide agents (Charudattan, 2001).

In general, microorganisms are very easy to work with, possess a short generation time (compared with insect herbivores), easy to deliver on plants and relatively cheap to produce on a large scale. Once a microorganism is applied to the weed, it becomes part of the ecosystem and regenerates on a seasonal basis to arrest any emergent weed, this however, makes it relatively cheap compared to chemical herbicides which have to be applied on a seasonal basis to a perennial weed such as water hyacinth. Most importantly, the fact that the environment (both aerial and edaphic factors) plays a role in plant pathogen interactions, it was deemed fit to investigate the potential of our native Nigerian fungi as mycoherbicides for water hyacinth control.

1.1 Statement of the Problem

Water hyacinth consistute a myriad of social menace to the economy as it impedes navigation on water bodies, promote good breeding conditions for mosquitoes, interferes with recreational use of water, fishing and transport operations; the major occupations of the riverine communities. It also interferes with hydroelectric power generation resulting in low level of industrialisation and trading activities respectively in Nigeria.

Management strategies of this aquatic weeds such as mechanical removal, the use of chemical herbicides and classical biological control measures (insect pest) have been found to be inadequate and expensive due to various factors hence, the global interest in the use of microbial measures. However, the success of microbial control measures is influenced by environmental factors. Unlike chemical herbicides which are environmentally unfriendly, mostly non-host specific and unaffected by environmental factors, imported microbial formulation (mycoherbicides) are known to be in Nigeria climatic conditions.

The goal of this work is therefore; to identify an indigenous fungal agent which is biologically active and stable in the southwestern part of Nigeria for use locally and regionally as a biocontrol agent for water hyacinth. This study will also determine the useful products obtainable from the organism for potential use in the control of water hyacinth in the areas of the world where the fungal agent cannot survive.

1.2 The Objectives of the Study

The Objectives of this Study are to:

i. Collect diseased samples of the plant and isolate fungi associated with the diseased plants.

ii. Evaluate the phytopathogenicity of the organisms on water hyacinth and to examine the histopathological changes on the infected water hyacinth leaf.

iii. Determine the host range specificity of the phytopathogenic isolate.

iv. Determine the best culture medium, best nitrogen source and optimum pH for the "in vitro" propagation of the phytopathogens.

v. Determine the fungal metabolite responsible for phytopathogenicity.

1.3 List of Abbreviations Used

Potato Carrot Agar (PCA).

Potato sucrose broth (PSB)

Potato Dextrose Agar (PDA)

Malt extract broth (MAB)

Potato Dextrose Broth (PDB)

Carboxymethyl cellulose (CMC)

Sabouraud Agar (SA)

Tap Water Agar (TWA)

Water Hyacinth Agar (WHA)

Water Hyacinth Leaf Broth (WHB)

Zapek-Dox Agar (ZA)

Potato sucrose Agar (PSA)

International Mycological Institute (IMI), now called CABI

Centre for Agriculture and Bioscience International (CABI)

International Monitoring Programme on *Echhornia crassipes* in Africa (IMPECCA)

CHAPTER TWO

2.0 LITERATURE REVIEW

2.1 Taxonomy of Water hyacinth

The English common names of the plant are waterhyacinth, water hyacinth, and water-hyacinth. *Waterhyacinth* is the standardized spelling adopted by the Weed Science Society of America (WSSA, 1984).

The taxonomic placement of waterhyacinth, based on Cronquist (1988), Thorne (1992), and Takhtajan (1997), is as follows: division Magnoliophyta; class Liliopsida; subclass Commelinidae (Liliidae [Cronquist, 1988; Thorne, 1992]); superorder Commelinanae (Thorne, 1992); order Pontederiales (Liliales [Cronquist, 1988]; Philydrales [Thorne, 1992]); family Pontederiaceae, genus *Eichhornia*; specific epithet *crassipes* (Martius) Solms-Laubach.

2.2 Biology of Water hyacinth

Waterhyacinth is an erect, free-floating, stoloniferous, perennial herb (Figure. 2.1). The bouyant leaves vary in size and morphology. The short, bulbous leaf petioles produced in uncrowded conditions provide a stable platform for vertical growth. Plants in crowded conditions form elongate (up to 1.5 m) petioles (Center and Spencer, 1981). Leaves are arranged in whorls of six to 10, and individual plants develop into Clones of attached rosettes (Center and Spencer, 1981).

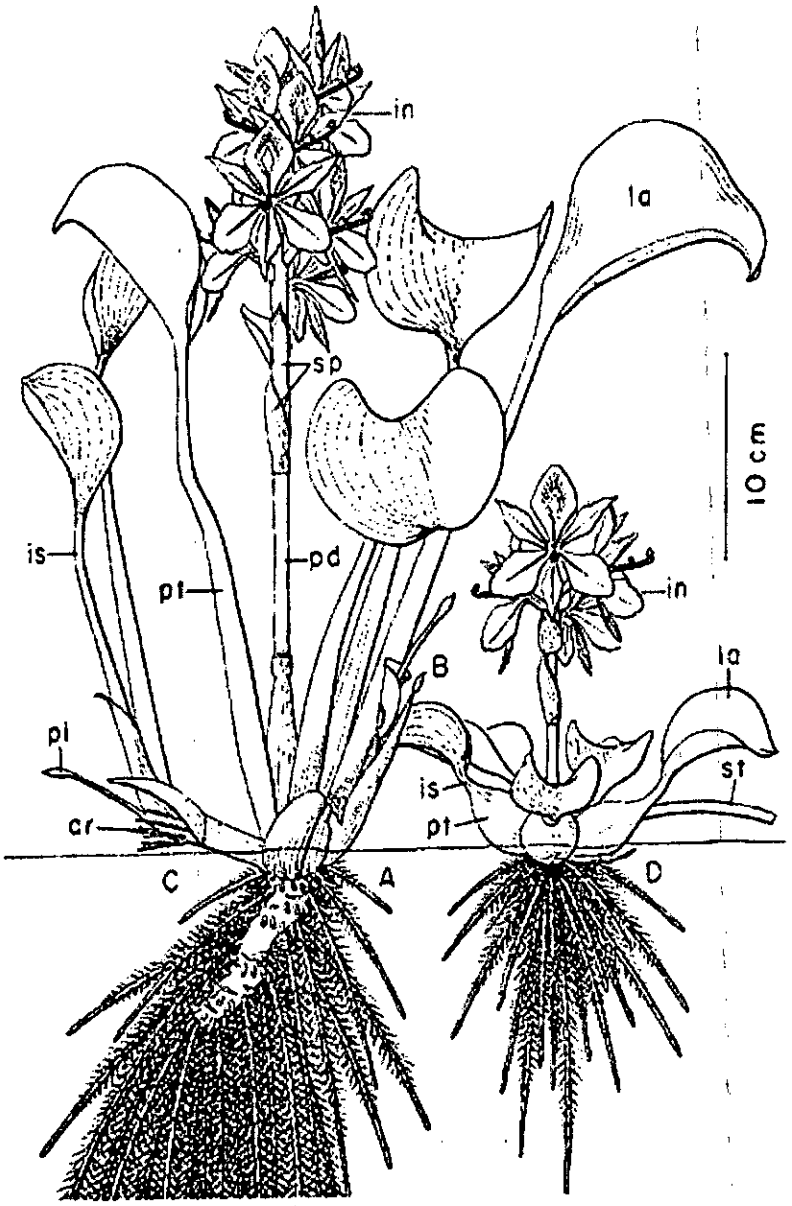

Figure 2.1. Morphology of waterhyacinth plants A. the "attenuated-petiole" rosette form produced in crowded conditions; B. an expanding axillary bud; C. a developing ramet; and D. the "bulbous-petiols" rosette form produced in open conditions. Abbreviations: ar- dventitious root; bb-bud bract; in-inflorenscense; is-lead isthmus; la-leaf blade; pl-primary leaf' pd-peduncle of flower spike; pt-leaf petiole; rh-rhizome; sp-spathe; st-stolon (Center *et al.*, 2002).

The lavender flowers display a central yellow fleck and are borne in clusters of up to 23 on a single spike (Barrett, 1980). The flowers may have short, medium, or long styles, but only the short- and long-style forms occur in the United States. The 14-day flowering cycle concludes when the flower stalk bends, positioning the spike below the water surface where seeds are released (Kohji *et al.*, 1995). Seed capsules normally contain fewer than 50 seeds each (Barrett, 1980). Each inflorescence can produce more than 3,000 seeds and a single rosette can produce several inflorescences each year (Barrett, 1980). The small, long-lived seeds sink and remain viable in sediments for 15 to 20 years (Center *et al.*, 2002). Seeds germinate on moist sediments or in warm shallow water (Center *et al.*, 2002) and flowering can occur 10 to 15 weeks thereafter (Barrett, 1980). Populations increase mainly by vegetative means.

Richards (1982), Watson (1984), and Watson and Cook (1982, 1987) describe waterhyacinth growth and population expansion as the result of differentiation of apical or axillary meristems. The single apical meristem on each stem tip can be vegetative, producing leaves with axillary buds, or reproductive, producing flowers. If an inflorescence develops, termination of the apical meristem halts leaf production. In this event, the axillary bud immediately below the inflorescence differentiates into a continuation shoot. This produces a new apical meristem that allows leaf production to proceed. If the axillary bud does not form a continuation shoot, then it produces a stolon. Elongation of the stolon internode moves the axillary bud apex away from the parent rosette. It then produces short internodes that grow vertically into a new rosette.

Waterhyacinth grows best in neutral pH, water high in macronutrients, warm temperatures (28° to 30°C), and high light intensities. It tolerates pH levels from 4.0 to 10.0 (Center *et al.*, 2002), but not more than 20 to 25% sea water (Muramoto *et al.*, 1991). The plants survive frost if the rhizomes do not freeze, even though emergent portions may succumb (Center *et al.*, 2002). Prolonged cold kills the plants, but reinfestation from seed follows during later warmer periods (Center *et al.*, 2002). Growth is inhibited at water temperatures above 33°C. Plants stranded on moist sediments can survive several months (Center *et al.*, 2002).

2.3 Related Native Plants of Water hyacinth

Waterhyacinth is a member of the pickerelweed family (Pontederiaceae). Families most closely allied with the Pontederiaceae are Commelinaceae, Haemodoraceae including Conostylidaceae (Takhtajan, 1997), Philydraceae, and Hanguanaceae (Hahn, 1997; APG, 1998). The subclass Commelinidae includes the Arecales, Poales, Commelinales, and Zingiberales (APG, 1998).

The Pontederiaceae is a small family of herbaceous monocotyledons that includes six genera and 30 to 35 species (Eckenwalder and Barrett, 1986). All seven members of the genus *Eichhornia* originated in tropical America, except for *Eichhornia natans* (P. Beauv.), which is from tropical Africa. Fourteen species of Pontederiaceae occur in the U.S./Canadian flora (Table 1), six of which are adventive; none are considered threatened or endangered (USDA, NRCS, 1999).

Table 2.1. Species of Pontederiaceae in the United States (Center *et al.*, 2002).

Native Species	Introduced Species
Heteranthera dubia (Jacq.) MacM.	*Eichhornia azurea* (Sw.) Kunth
Heteranthera limosa (Sw.) Willd.	*Eichhornia crassipes* (Mart.) Solms.
Heteranthera mexicana Wats.	*Eichhornia diversifolia* (Vahl) Urban
Heteranthera multiflora (Griseb.) Horn	*Eichhornia paniculata* (Spreng.l) Solms
Heteranthera penduncularis Benth.	*Monochoria hastata* (L.) Solms
Heteranthera reniformis Ruiz Lopez & Pavon	*Monochoria vaginalis* (Burm. f.) K. Presl
Heteranthera rotundifolia (Kunth) Griseb.	
Pontederia cordata L.	

2.4 Historical Background of Water Hyacinth

Stories about the introduction of the water hyacinth plant to different countries range from mythical tales to pure accidents (Khan and Thyagarajan, 1988). The plant is believed to have been introduced first into Africa in Egypt between 1879 and 1892. The plant appears to have been introduced into Sri Lanka as an ornamental plant in 1904. During 1930 it became a serious weed. A Japanese exhibitor coming to the 1884 cotton exposition in New Orleans, Louisiana, brought along this plant because of its attractive flower. They had collected the plant from Orinoco River in Venezuela. These were given away at the exposition as souvenirs.

It became problem in Southern United State when many of these were tossed into drainages, canals, swamps and streams. Because of the lack of natural enemies such as insect, virus etc, to keep this plant in check in its native environment, water hyacinth grew explosively. The innocent gesture caused infestation in Florida, Louisiana and Texas resulting in a yearly budget of US$11million for the three states as far back as 1985 (Khan and Thyagarajan, 1988).

The breeding rate of this plant is phenomenal. Studies have shown that two water hyacinth parents produced 300 offspring in 23 days and 1,200 within 4 months. It was reported that under favorable condition 10 plants can multiply to 600,000 and virtually carpet an acre of water in 8 months (Khan and Thyagarajan, 1988).

Report shows that in 1957, the Belgian Congo spent 1.5 million dollars in an eradication programme while the Sudan spent approximately 2.5 million dollars since 1963 (Khan and Thyagarajan, 1988).

Despite all these, the plant persists today and continues to be the number one aquatic problem in the world.

2.5 Pest Status of Weed of Water Hyacinth

Waterhyacinth, *Eichhornia crassipes* (Mart.) Solms.-Laubach (Figure 2.2), is considered one of the world's worst weeds (Holm *et al.*, 1977), invading lakes, ponds, canals, and rivers. It was introduced into many countries during the late 19th and early 20th centuries, where it spread and degraded aquatic ecosystems. It is still rapidly spreading throughout Africa, where new infestations are creating life-threatening situations as well as environmental and cultural upheaval (Center *et al.*, 2002). Control with herbicides, particularly 2,4-D, is feasible, but is costly, and problematic in the environment.

Figure 2.2. Waterhyacinth growing in the Marañon River, Peru. (Photo by T.D. Center. USDA, ARS.)

Figure 2.3. A waterhyacinth infestation in southern Florida. (Photo by T.D. Center, USDA, ARS.)

2.6 Nature of Damage by Water Hyacinth

2.6.1 Economic damage

Waterhyacinth grows rapidly forming expansive colonies of tall, interwoven floating plants. It blankets large water bodies (Fig. 2.3), creating impenetrable barriers and obstructing navigation. Floating mats block drainage, causing flooding or preventing subsidence of floodwaters. Waterhyacinth hinders irrigation by impeding water flow, by clogging irrigation pumps, and by interfering with weirs (Center *et al.*, 2002). Multimillion-dollar flood control and water supply projects can be rendered useless by waterhyacinth infestations (Center *et al.*, 2002).

Infestations block access to recreational areas and decrease waterfront property values, oftentimes harming the economies of communities that depend upon fishing and water sports for revenue. Shifting waterhyacinth mats sometimes prevent boats from reaching shore, trapping the occupants and exposing them to environmental hazards (Harley, 1990). Waterhyacinth infestations intensify mosquito problems, increases habitat for

species that attach to plants, and impedes runoff and water circulation (Center *et al.*, 2002).

2.6.2 Ecological damage

Dense mats reduce light to submerged plants, thus depleting oxygen in aquatic communities. The resultant lack of phytoplankton alters the composition of invertebrate communities, ultimately affecting fisheries (Center *et al.*, 2002). Higher sediment loading occurs under waterhyacinth mats due to increased detrital production and siltation. Herbicidal treatment or mechanical harvesting of waterhyacinth often damages nearby desirable vegetation.

2.6.3 Extent of losses

Waterhyacinth caused annual losses of $65 to 75 million in Louisiana during the 1940s (Center *et al.*, 2002). Fish and wildlife losses alone in the six southeastern states exceeded $4 million per year in 1947 and waterhyacinth control provided a benefit to cost ratio of 15.3:1 (Center *et al.*, 2002). A total loss of $43 million in 1956 was ascribed to waterhyacinth infestations in Florida, Mississippi, Alabama, and Louisiana (Center *et al.*, 2002). The U.S. Army Corps of Engineers estimated benefits from waterhyacinth control programs at nearly $14 million in 1965 (Center *et al.*, 2002). Florida spent more than $43 million during 1980 to 1991 to suppress waterhyacinth and waterlettuce (Schmitz *et al.*, 1993). The annual costs for waterhyacinth management range from $500,000 in California to $3 million in Florida (Mullin *et al.*, 2000).

2.7 Nigerian Experience of Water Hyacinth

Over the last 100 years water hyacinth, *E. crassipes* (Martius) Solms - Laubach, has spread from its native range in Central and South America to tropical and subtropical regions throughout the world via natural lakes, water courses and man made impoundment.

The resulting infestations caused greater problems than those of any other floating aquatic weed, particularly in North America, Africa India, South East Asia and Australia. It forms dense impenetrable mats in natural water bodies. It impedes boat traffic, the recreational use of water and fishing, and it enhances the breeding of vectors of human and animal diseases.

The attention of the Nigerian public was first drawn to the water hyacinth invasion of the Lagos Lagoon by the Nigerian Television Authority (NTA), Lagos on 9th January, 1985 (Ogunye, 1988).

The response from the Nigerian scientists was quite prompt and encouraging as they identified the plant as water hyacinth and alerted the nation on its dangerous nature and the economic loss if kept unchecked on our water. On the basis of this information the NTA then sent a team of scientists to Sudan and Egypt for verification (Ogunye, 1988).

The report submitted by these scientists to the Federal Ministry of Science and Technology also prompted Government to inaugurate an Inter-ministerial committee on the 7th March, 1985 (Ogunye, 1988).

Membership included representatives from Federal Ministries, Universities, Research Institutes; State Governments, the private sector and the Armed forces and the chairman was Dr. Olu Ogunye. To facilitate its activities, inter-ministerial committee had to group

its members to subcommittees such as; subcommittee on clearance and surveillance, the research subcommittee and the public enlightenment sub-committee.

Investigations by inter-ministerial committee later confirmed that the weed was carried by water current into Nigeria from the neighbouring Republic of Benin, arriving Badagry around September 1984 and reaching the Lagos Lagoon in January 1985 (Ogunye, 1988). Series of tours by road, boat and air were undertaken by this committee. With the assistance of the Nigerian Air Force, aerial photographs were taken. With the heavy infestation in the country, the immediate option opened was the physical removal using the local fishermen and their boats. The first nominal clearance of the inlets and parts of the Badagry creek took place from June to August 1985 (Ogunye, 1988).

The committee realized the need to import mechanical harvesters from oversea but could not do so due to none availability of funds. In view of this, 10 locally fabricated harvesters were made and in 1987 the Canadian Government gave 2 harvesters through her embassy in Lagos which were launched in April same year.

The 1986/87 surge came in December 1986 and a private company that had come up with an effective improvised harvester was promptly employed to handle the clearance operation.

During this period, strands of water hyacinth were able to escape the salt water of the Lagos Lagoon into the fresh waters of Ilaje, Okitipupa in Ondo State via Epe (Lagos State). This moved forward and appeared in Edo State in June 1987 (Ogunye, 1988).

The infestation caused serious anxiety among governments and people of the affected states.

The riverine inhabitants that depend mainly on fishing for their livelihood and whose only transportation route is the water were the worst affected. Fish then became expensive in the market generally. Villages' children could not go to school in the neighbouring towns neither could they take their sick to the hospital. Therefore, a number of deaths were reported in these areas due to lack of access to prompt medical care. Trading and shopping with neighbouring towns were adversely affected. The patrol boats of Law Enforcement officers could not operate, while the floatation of logs for the timber industry was terribly hampered. The adverse effects experienced by the people and the economy cannot be imagined (Ogunye, 1988). The Sub-committee also enjoined the public to join hands with the government in controlling the weed. Right from the on-set, the inter-ministerial committee had recognized the importance of research in finding lasting solution to the water hyacinth problem. The committee had therefore constituted a powerful research team headed by Professor B.A Oso of the University of Ibadan who was also the national research coordinator of the water hyacinth project. Members of the Research Sub-Committee were drawn from the Universities and Research Institutes in the country. At that time the research efforts were concentrated on seven areas of priority. These were:

(a) Designing and fabrication of suitable mechanical devices locally for harvesting the weed

(b) Studies on the biology and the ecology of water hyacinth;

(c) Hydro-Biological studies of the creeks and lagoons infested by the weed;

(d) Chemistry and Biochemistry of the weed;

(e) Economic uses of the weed;

(f) Chemical control; and

(g) Biological control of the weed.

Each research area was handled by group of scientist, despite the fact that only limited amount of money was made available for research, the sub-Committee made appreciable progress as briefly highlighted below.

(a) **Designing and fabrication of harvesting devices:** The committee mandated the Nigerian Institute of Oceanography and Marine Research (NIOMR) to design and fabricate harvesting devices and barriers using the local raw materials. The institute eventually came up with a dragnet for harvesting, and two floating barriers for containing the weed. When tested the dragnet was found to be effective in harvesting mid-stream floating water hyacinth. It was unable to operate against thick mats of the weed. The floating barriers were found to be effective in holding back the weed for immediate clearance operations. However, the committee, realizing that a harvesting device that actually rakes and collects would be more effective, mandated NIOMR once more together with a number of research institutes and Universities to work together to design and fabricate a harvester that can do this. Also, two indigenous companies were able to come up with locally improved mechanical harvesters which were found very effective.

(b) **Biological and Ecology of water hyacinth**: The group was headed by Professor Kola Kusemiju of the University of Lagos. Studies here covered the effect of environmental factors on the survival, growth and reproduction of the weed. Factors considered include, among others, salinity and pH tolerance of the weed, seasonal variation of the weed mass and decomposition of dead weeds. The results obtained showed that the water hyacinth shows a wide variation in salinity tolerance.

(c) **Hydro-Biological Studies:** The group was headed by Professor A. B. M Egborge of the University of Benin. It is a known fact that salinity, temperature and level of pollution of any given body of water play a major role in water hyacinth infestation, the weed being a predominantly fresh water plant. Hence it was considered of importance to carry out studies on the condition of waterways around Badagry and Lagos where the invasion first occurred. This was done over a period of one year in order to detect any annual variations. Between September 1986 and September 1987 monthly water samples were taken from a number of stations in the creeks between Lagos harbours and Yewa Lagoon and the Nigerian /Benin Republic border. The parameters determined included air temperature, water temperature, water transparency and salinity. It was shown, among other things that the period of abundance of water hyacinth generally coincided with the periods of low salinity.

(d) **Chemistry and Biochemistry of water hyacinth:** The group was headed by Professor O.L Oke of Obafemi Awolowo University, Ile Ife.

For the economic uses of water hyacinth particularly as animal feed, it was considered necessary to know its nutrient level and chemical composition. The root, rhizomes and leaves of the plant were analysed for protein, tannins and mineral salts and screened for alkaloids, saponins, glycosides and anthroquinones. The results obtained showed that the leaves contain a good amount of extractable protein which makes the plant suitable as animal feeds.

(e) **Economic uses of the water hyacinth:** The group is headed by Dr A.C Ibe of the Nigerian Institute of Oceanograph and Marine Research (NIOMR), Lagos.

Studies were carried out to determine the suitability of the water hyacinth as fish feed. After determining the chemical composition of the various part of the plant it was processed into fish feed and subsequent feeding experiments showed that water hyacinth could suitably be used in fish feed formulations.

The scientists at the Federal Institute of Industrial Research, Oshodi (FIIRO) succeeded in pulping water hyacinth and obtaining from it medium density fibreboards suitable for ceiling and panel work.

(f) **Chemical control of the water hyacinth**: The group was headed by Professor A.M.A Imevbore of Obafemi University Ile-Ife. Five herbicides, diquat, paraquat, terbutryn, 2,4-D and glyphosate were shown to attack and kill the weed. Even though chemical control of water hyacinth was possible, the main objections to its use in the Nigerian situation were:

i. The rural populations often use creeks, Lagoons and rivers for domestic and drinking purposes in view of lack of pipe borne water.
ii. It causes serious pollution problems as the weed decay.
iii. Lagoons and rivers are the major sources of fishing nursery grounds for early development of fishes and shrimps. Such aquatics may be contaminated.
iv. Unforeseen toxic effects of residual chemicals on aquatics and man.
v. The chemical drifts to agricultural crops and its non specific nature to water hyacinth.

(g) **Biological control of the water hyacinth:** This group was headed by Professor B.A Oso of the University of Ibadan.

Biological control is recognized as probably the most effective, economical, long term environmentally safe control method for the water hyacinth. It is known that the water

hyacinth is not much of a problem in its region of origin in South American because there exists in that area natural enemies of the weed, e.g insects and fungal pathogens, that are constantly attacking it and keeping its population low. Exploration visits are usually undertaken by scientists from other troubled countries to that region for collection of such organisms for use in controlling the water hyacinth in their countries. However, here in Nigeria, the group succeeded in obtaining six fungal pathogens from the water hyacinth which have been found to attack and destroy the plant.

2.8 Pattern of Water Hyacinth Distribution in Western Nigeria

The following pattern of water hyacinth distribution was recorded in Nigeria in 1969 (Kusemiju, 1988).

i) Badagry Creeks

The creeks are 177km long and include Yewa and Ologe lagoons. Active fisherman in the creeks number about 2500, operate 1380 single canoes and account for about 8% of the inland fish catches of the lagoon system. The major ecological factors operating in the creeks have been documented by Ezenwa and Kusemiju (1984). Yewa Lagoon has since become a major depository area of water hyacinth along the Badagry Creek. Annual surge of water hyacinth now occur regularly from the Yewa Lagoon following each rainy season when the main stream of the water flows into the main Badagry Creek en-route to Lagos.

ii) Lagos Lagoon

This lagoon has a surface of about 208 km^2 and its waters exhibit seasonal salinity gradients. The lagoon experiences high brackish water condition during the dry season (December-May) with salinity as high as 28.9%. Freshwater condition occurs in the rainy

season (June to November) with salinity as low as 1%. Salinity is thus a major limiting factor to the growth of water hyacinth in the Lagos lagoon. If rechecked, the lagoon is likely to continue to experience surge of water hyacinth from the Badagry Creek, Festac Lake and adjacent lagoons eastwards of Lagos as the weed flows into the sea through the Lagos Harbour.

iii) Epe Lagos

This lagoon is connected to the Lagos lagoon and has a total coverage of 234 km^2. There are over 2300 single canoes operating in the area and account for 36% of the inland catches. While the low brackish water conditions suitable for growth of water hyacinth occur from the Palaver Island to Epe town, the heavy rains each year together with the current carry the weeds into the Lagos Lagoon. Thus, the Epe Lagoon is a major transit for the weeds into the Lagos Lagoon.

iv) Lekki Lagoon

This Lagoon has become a major water hyacinth depository in Nigeria. It has a surface area of 247 km^2. It supports a major fishery with as many as 30 fishing villagers/settlements settled on the edge of the lagoon (including Origbe, Dopamu, Lupaye, Imoba, Emina). There are well over 2000 fishing canoes operating in the lagoon in addition to draw net and sudding cutting fishermen account for nearly 10,000 active fishermen. The lagoon is completely fresh water, the highest salinity obtained in the lagoon being only 0.3% (Kusemiju, 1981). This lagoon has become the main centre for water hyacinth proliferation in Nigeria. For example in June 1988, nearly 70% of the 247 km^2 surface area of the lagoon was covered by water hyacinth with most of the fishing and river transportation within the lagoon being hampered.

v) Central Creeks in Ondo State

The Central Creeks in Ondo State which have been injured by the weed include the Akata Creek and Mape River. The Creeks is about 130 km^2 long and together with the surrounding streams provide ideal condition for the growth of water hyacinth. Alape Creek supports a major Heterotis fishery and is known to be rather deep (over 35 m in major area) but is now covered with luxuriant growth of the weed.

vi) Mahin Lagoon

The Mahin Lagoon is another major depository for the water hyacinth. It is freshwater and very shallow. From here, water hyacinth is normally carried by heavy wave action and distributed to the entire Creeks as far afield as 400 km away. Since the Mahin Creek forms the link between the riverine people of Ondo State and the rest of the country, the people living around the Creek have virtually been cut off from the rest of the country by the thick growth of the weed in the Lagoon.

2.9 Biological Control Agents of Water Hyacinth

One of the measures employed world-wide to check the menace of water hyacinth is biological control, using organisms that attack and destroy the weed. In the region of origin of water hyacinth in the Amazon Basin in South America, the weed is not much of a problem because, naturally there are organisms which attack the weed and keep its population much below the nuisance level. Scientists have therefore taken advantage of this by evolving biological control measures using these natural enemies of the weed. Explorative studies in the Neotropics for potential control agents of water hyacinth have shown a good number of organisms capable of attacking water hyacinth and considerably reducing its vigour. Those found to impact the weed effectively and which have been shown to be host specific to it have since been used in controlling the weed in many countries. Among these are some phytophagous insects (*Neochetina eichhorniae* and *N. bruchi*) and fungal pathogens (*Acremonium zonatum, Rhizoctonia solani, Alternaria alternate* and *Cercospora* sp.) (Oso, 1988).

2.9.1 Some Promising Phytophagous Insects of Water Hyacinth

2.9.1.1 *Neochetina eichhorniae* and *N. bruchi* (Coleoptera: Curculionidae)

Members of the genus *Neochetina* are semiaquatic weevils that feed only on species of Pontederiaceae. Center (1994) reviewed the biologies of *N. eichhorniae* and *N. bruchi.*

Adult feeding creates characteristic rectangular scars on the leaves, about 2 to 3 mm in width and of variable length, sometimes girdling the leaf petioles at the distal end and causing the blade to dessicate (DeLoach and Cordo, 1983; Wright and Center, 1984;

Center *et al.*, 1999a). Moderate to severe weevil infestations cause plants to be shorter with smaller leaves, fewer offsets and flowers, lower tissue nutrient content and reduced overall vigor than uninfested or lightly infested plants (Center and Van, 1989).

2.9.1.2 *Eccritotarsus catarinensis* (Heteroptera: Miridae)

Eccritotarsus catarinensis is a leaf-sucking bug (2 to 3 mm long). Eggs are inserted into the leaf tissue parallel to the surface and the four nymphal instars feed gregariously with the adults on the underside of the leaves, causing severe chlorosis. Development of the eggs and nymphs requires 23 days and adults live 50 days (Hill and Celliers., 1999).

E. catarinensis has been released in China and imported into Thailand for pre-release evaluation (Center *et al.*, 2002).

Eccritotarsus catarinensis is now established in South Africa (Hill and Cilliers, 1999). The insect reaches very high densities in tropical areas of the country where it is capable of causing severe die back of the water hyacinth plants.

2.9.1.3 *Niphograpta albiguttalis* (Lepidoptera: Pyralidae)

The small (ca. 0.3 mm), spherical, and creamy-white eggs of *N. albiguttalis* take three to four days to hatch at 25°C. Larval development requires about two weeks. The fully-grown larva excavates a cavity in a healthy leaf petiole, in which it forms its cocoon. Pupation occurs in the cocoon and the pupal stage lasts seven to 10 days. The emerging adult moth exits the petiole through a silken tunnel prepared by the larvae before pupation (Center *et al.*, 2002).

2.9.1.4 *Orthogalumna terebrantis* (Acarina: Galumnidae)

The waterhyacinth mite, *O. terebrantis*, like other mites, has piercing mouthparts with which it sucks plant juices. Its host plants include pickerelweed and waterhyacinth (Center *et al.*, 2002). Feeding damage of this mite is restricted to the leaf blades. Larval feeding causes small reddish spots on the leaf surface and the nymphs produce galleries that extend about 6 mm towards the apex. The adults emerge through round exit holes at the end of the gallery.

Large mite populations produce up to 2,500 galleries on a single leaf, which desiccate the blade (Center *et al.*, 2002). Severe damage is usually localized or confined to a few plants but, when combined with other stresses, it can contribute to declines (Center *et al.*, 2002).

2.9.1.5 *Xubida infusellus* (Lepidoptera: Pyralidae)

Pyralid *X. infusellus* is considered to be one of the most important phytophagous species on waterhyacinth in South America. The larvae severely damage leaf petioles and can destroy shoots by feeding on apical meristems and burrowing into rhizomes. The damage is similar to that of *N. albiguttalis* or *B. densa* (DeLoach *et al.*, 1980). *Xubia infusellus* prefers advanced phenostage plants with elongate leaf petioles (see Center *et al.*, 1999a), whereas *N. albiguttalis* prefers younger plants with inflated leaf petioles.

The life history of *X. infusellus* is well documented by DeLoach *et al.* (1980) and Sands and Kassulke (1983). First instar larvae briefly feed externally, sometimes girdling a petiole before entering it, but then feed internally. They burrow downward, sometimes transferring to adjacent leaves, until they eventually encounter the rhizome. Late instar

larvae form large burrows, causing extensive damage. Larvae cut emergence holes in the petiole prior to pupation that they close with silk, and then pupate just below the covered opening. The total developmental requires 64 days at 26°C (Sands and Kassulke, 1983). The adult lives four to eight days (Sands and Kassulke, 1983).

This insect has been established in Australia (Julien and Griffiths, 1998). It was also released in Papua New Guinea (Julien and Stanley, 1999).

2.9.1.6 *Thrypticus* spp. (Diptera: Dolichopodidae)

Thrypticus species are all phytophagous stem miners of monocots in the Cyperaceae, Graminiaceae, and Juncacaeae. Females possess a characteristic sclerotized, blade-like structure used to pierce stems in preparation for oviposition. *Thrypticus* species has been associated with waterhyacinth in Trinidad, Guyana, Surinam, and Brazil, Belize, Jamaica, Barbados, Argentina, Uruguay, Northern South America (Center *et al.*, 2002).

Females lay eggs singly in young petioles of *E. crassipes*, inserting eggs into the tissues, usually just above the water line. Petioles are suitable for oviposition only when recently separated from the sheath; thus all galleries in an individual petiole are of similar age. Larvae continue to feed in galleries, which they enlarge and lengthen.

When petioles have large numbers of larval galleries, damage can be extensive. Mitchell It has been noted that nearly all plants attacked at Santos, Brazil, showed extensive rotting of petioles bases and, in many cases, had completely collapsed (Center *et al.*, 2002).

Thrypticus have been found attacking *E. crassipes, E. azurea, P. cordata*, and *Pontederia rotundifolia* L. in northern Argentina (Center *et al.*, 2002).

2.9.1.7 *Cornops aquaticum* (Orthoptera: Acrididae)

The grasshopper; *C. aquaticum* has been considered to be among the most damaging of the South American insects associated with waterhyacinth. Despite heavy egg predation by the weevil *Ludovix fasciatus* (Gyllenhal), *C. aquaticum* is abundant and very damaging. Its broad distribution from Argentina through Mexico indicates that it can tolerate a wide range of climatic conditions. However, concern over its host specificity has precluded consideration for release in the United States. This oligophagous species is clearly not suitable for release in the United States (Center *et al.*, 2002).

This organism's total development requires about 50 days. Adults live up to 110 days, and are mobile, strong fliers, and extremely damaging to the plant.

C. aquaticum has been observed on *E. azurea, P. cordata, P. rotundifolia* and *Pontederia subovata* (*Seub. in Markt.*) *Lowden*, in addition to waterhyacinth (Center *et al.*, 2002).

2.9.2 Establishment and Spread of Phytophagous Insects

Neochetina eichhorniae was released in southern Florida in 1972, using eggs from 2,479 adults sent from Argentina during August 1972 to March 1973. Adults removed from founder colonies were then redistributed by numerous agencies. As a result, *N. eichhorniae* was released at 199 sites in Florida, 492 sites in Louisiana, one site in Texas, and four sites in California (Cofrancesco, 1984, 1985). However, *N. eichhorniae* was

already present when initial releases were made in Texas, having apparently dispersed from southern Louisiana, and by 1984 it was at several waterhyacinth infestations between Port Arthur and Corpus Christi (Cofrancesco, 1984). Large numbers of weevils, many actively flying, were observed at nights in southern Louisiana during 1980 (Center, 1982), clearly indicating a capacity to disperse.

When *N. bruchi* became available, there was no similar dissemination campaign. As a result, it was released at only 40 sites: 21 in Florida, 10 in Louisiana, five in Texas, and four in California (Stewart, 1987). Despite this disparity in release efforts, both species are now ubiquitous in Florida (Center and Dray, 1992; Center *et al.*, 1999a).

Niphograpta albiguttalis was initially released only in southern Florida, but populations dispersed more than 500 km within 18 months (Center *et al.*, 2002). This moth was released at two sites in Louisiana during May 1979 and collected 27 km from the nearest release site a year later (Center *et al.*, 2002). *Niphograpta albiguttalis* appeared to be absent from Texas in 1985, and so was released at a few sites during May 1986. It was widely dispersed by July 1986 (Stewart, 1987), probably originating from Louisiana. This insect was never released in Mexico but was found there (Julien and Griffiths, 1998). So it is likely that these populations were derived from the ones in the United States, with the nearest release site being about 1,600 km away. Likewise, although there are no recorded of releases of *N. albiguttalis* in Puerto Rico (Julien and Griffiths, 1998), larvae were collected near San Juan in 1995 (Center *et al.*, 2002).

2.9.3 Efficacy of Phytophagous Insects on Water hyacinth

Numerous field studies document the decline of waterhyacinth in diverse geographical areas of the United States after introductions of biological control agents (Cofrancesco, 1985; Cofrancesco *et al.*, 1985; Center and Durden, 1986). Waterhyacinth now occupies one-third of its former acreage in the Gulf Coast states (Cofrancesco *et al.*, 1985; Center *et al.*, 1990). This reduction resulted from both direct plant mortality and reduced regrowth after winter diebacks, perhaps along with reduced flowering and seed production (Center *et al.*, 1999a, b). Feeding by insects destroys meristematic tissue causing the plants to lose their ability to replace senescent tissue. They then lose bouyancy and sink. Often, they merely stop growing due to the destruction of axillary buds and reduced carbohydrate reserves.

The most recent and most spectacular effects of the waterhyacinth weevils have occurred at Lake Victoria in East Africa. Waterhyacinth was first recorded on the lake in 1980 and by the mid-1990s, some 12,000 ha of the weed were clogging bays and inlets. Uganda made the first introductions of *N. eichhorniae* and *N. bruchi* in 1995, followed by Kenya and Tanzania in 1997 (Center *et al.*, 2002). A significant reduction in the extent of the weed on the Ugandan shore was evident by November 1998, with many of the mats having sunk. These results were later repeated on the Kenyan and Tanzanian shores. An estimated 75% of the mats on the Kenyan side had sunk by December 1999 (Center *et al.*, 2002). The spectacular results of the biological control program on Lake Victoria using the two weevil species are the same as those achieved on Lake Kyoga (Uganda) and on the lagoons of the Sepik River (Papua New Guinea) (Center *et al.*, 2002). Similar results

have been obtained in Sinaloa, Mexico where the release of *N. eichhorniae* and *N. bruchi* during 1995 to 1996 reduced 3,041 ha of waterhyacinth distributed over seven impoundments by 62% to 1,180 ha (Center *et al.*, 2002).

2.9.4 Promising Fungal Agents for Biocontrol of Water Hyacinth

There are several good reasons to consider fungal pathogens as biocontrol agents: pathogens can cause significant reductions in water hyacinth biomass, especially following natural disease outbreaks, after severe insect attacks, or when used as inundative bioherbicide agents (Charudattan *et al.*, 1985; Shabana *et al.*, 1995b). Reports of natural disease outbreaks further confirm the potential of pathogens in limiting water hyacinth populations (Martyn, 1985; Morris, 1990). Controlled experiments have confirmed the potential of fungi to control water hyacinth (Martyn and Freeman 1978; Charudattan *et al.*, 1985; Shabana *et al.*, 1995b). In addition, it has been well proven that pathogens can be successful as classical or inundative (bioherbicide) agents. Currently, several weeds are biologically controlled with pathogens (Morin *et al.*, 2006). Of the nearly 70 fungi and bacteria recorded on water hyacinth, only about 15 have been adequately tested and confirmed to be highly virulent pathogens (Barreto and Evans, 1996; Charudattan, 2001). Of these, three fungal pathogens, *Acremonium zonatum, Alternaria eichhorniae,* and *Cercospora piaropi (= C. rodmanii)*, have been studied intensively as biocontrol agents and shown to be effective in controlling water hyacinth under experimental conditions (Martyn and Freeman, 1978; Charudattan *et al.*, 1985; Shabana *et al.*, 1995b). This leaves a large number of other reported fungi and bacteria to be assessed for their biocontrol potential. Thus, for now, the choice of pathogens for biological control is limited to classical biocontrol and bioherbicide agents, *Uredo*

eichhorniae, A. zonatum, A. eichhorniae, C. piaropi (= C. rodmanii), Myrothecium roridum, and *Rhizoctonia solani* and several others, less widely distributed pathogens, such as species of *Bipolaris, Drechslera,* and *Fusarium,* which may hold promise, but need to be studied further to confirm their potential.

2.9.5 Some Highly Virulent and Useful Pathogens

The following are brief descriptions of virulent pathogens that are leading candidates for further development in water hyacinth control.

2.9.5.1 *Acremonium zonatum*

This fungus was reported by Charudattan (2001) to cause an easily identified necrotic zonate leaf spot characterised by spreading lesions, most noticeable on the upper laminar surface. In the lower surface, which is normally protected from direct sunlight, the area directly under the spot may have a sparse, spreading layer of white fungal (mycelial) growth. Each spot may be small (2 mm diameter) to large (> 3 cm diameter) and the spots may coalesce, covering most of the lamina. The zonate pattern may not be evident in new infections when most spots are small. This disease has been reported from Australia, USA, and many countries of Asia, Central America, and South America (Charudattan, 2001).

2.9.5.2 *Alternaria eichhorniae*

Alternaria eichhorniae has been reported to be a potentially good bioherbicide agent (Shabana *et al.,* 1995a, b). It causes discrete necrotic foliar spots (oblong, 2–4 mm long) surrounded by a bright yellow halo. In culture, *A. eichhorniae* produces several bright red compounds in culture, including bostrycin and deoxybostrycin that are phytotoxic to

water hyacinth leaves (Charudattan and Rao, 1982). The organism has been recorded on water hyacinth in Australia, Bangladesh, Egypt, India, Indonesia, and South Africa.

2.9.5.3 *Cercospora piaropi* (= *C. rodmanii*)

These strains have been reported by Charaudattan (2001) to cause small (2–4 mm diameter) necrotic spots on laminae and petioles of water hyacinth. The spots are characterised by pale centres surrounded by darker necrotic regions. Occasionally, the spots may appear in the shape of 'teardrops' that coalesce as the leaf matures, causing the entire leaf to turn necrotic and senescent. The senescence is accelerated by the *Cercospora* disease, and the disease can rapidly spread across water hyacinth infestations, causing large areas of the weed mat to turn brown and necrotic. Under severe infections, the plant may be physiologically stressed, lose its ability to regenerate, become waterlogged, and sink or disintegrate (IMPECCA Technical Guide Series 1, 2001; Charudattan 2001). The isolates have been reported on water hyacinth in USA, Mexico, Venezuela, Brazil, South Africa, India, Malaysia, Egypt and Zambia (IMPECCA Technical Guide Series 1, 2001; Charudattan 2001).

2.9.5.4 *Myrothecium roridum*

This fungus causes a teardrop-shaped leaf spot (up to 1 × 5 cm), rounded on the side facing the petiole and tapering to a narrow point in the direction of the laminar tip. Older leaf spots turn necrotic with dark brown margins, with the centre of the spot covered with discrete white and black conidial masses. Myrothecium disease of water hyacinth has been reported to occur in India, Malaysia, Indonesia and Mexico (IMPECCA Technical Guide Series 1, 2001; Charudattan 2001). Some studies suggest that some *Myrothecium* species can be used as broad-spectrum bioherbicides against several weeds (Walker and

Tilley, 1997), a finding that has implications for the development of *M. roridum* for water hyacinth control.

2.9.5.5 *Rhizoctonia solani*

The disease symptoms caused by this fungus may resemble damage caused by a desiccant type of chemical herbicide (e.g. diquat). Symptoms consist of irregular, necrotic spots and broad lesions. Unlike chemical damage, the brown necrotic areas are usually surrounded by noticeable, thin, water-soaked margins of darker brown colour than the rest of the lesion (IMPECCA Technical Guide Series 1, 2001; Charudattan 2001). Rhizoctonia disease has been reported on water hyacinth from the southeastern United States, Brazil, Mexico, Panama, Puerto Rico, India, Malaysia, and Indonesia (IMPECCA Technical Guide Series 1, 2001; Charudattan 2001). This fungus is usually very aggressive and destructive, capable of rapidly killing water hyacinth plants. The extent of variability in virulence of *R. solani* pathogenic to water hyacinth is not clear, but isolates collected in the USA, Panama, and Brazil have been found to be extremely virulent (Bateman, 2001).

2.9.5.6 *Uredo eichhorniae*

This rust fungus occurs in southern Brazil, Argentina, and Uruguay. It is known only in its uredial spore stage. It is a rust fungus, and it is likely to be highly host-specific (Charudattan 2001) and therefore a desirable classical biological control agent, but several aspects of its biology remain to be fully understood (Charudattan, 2001). It is unclear why it does not occur beyond its present range of distribution in the subtropical to temperate regions of South America. The addition of this rust pathogen to the existing

suite of biocontrol agents is likely to improve the prospects for a sustainable, long-term biological control of water hyacinth (Bateman, 2001).

2.10 Phytotoxins in Plant Disease

Typical symptoms of most plant diseases revealed the involvement of phytotoxic metabolites, which therefore suggest a role for toxic metabolite secreted by the pathogen in the disease development. Metabolites of many fungi may have adverse or stimulatory effects on plants such as suppression of seed germination, malformation, and retardation of seedling growth (Lynch and Clark, 1984; Heisey *et al.*, 1985; Rice, 1995). Neergaard (1979) reported that some fungal pathogens often produce phytotoxins that affect seed germination and seedling growth. Betina (1984) reported that some fungi on the surface of seeds often produce mycotoxins that affect food quality. Tey-Rulh *et al.*, (1991) reported that the foliar symptoms of Eutypa-infected grapevines are that of the toxin, eutypine, produced by the fungus and not the direct result of the fungus. Plant pathogenic organisms produce toxins that are often related to pathogenicity or virulence and cause various symptoms including necrosis, chlorosis, wilting, water soaking and eventually the death of the host plants (Scheffer, 1983).

Phytotoxic metabolites produced by pathogenic organisms have been found to produce symptoms similar to those caused by that pathogen. Such toxic metabolites include pinolidoxin from *Ascochyta pinodes*, deoxyradicin and maculosin from *Alternaria helianthi* and *Alternaria alternata*. Identified metabolites from other pathogens include piricularin from *Piricularia oryzea*, victorin from *Cochliobolus vitoriae*, phaseolotoxin from *Pseudomonas syringae* pv *phaseolicola*, saccharitoxin from *Helmithosporium sacchari*, cercosporin from *Cercospora* spp. (Amusa, 2006). Phytotoxic metabolites of

most of these pathogens have been reported to play a significant role in pathogenesis (Amusa *et al.*, 1993).

2.10.1 Microbial Toxin Production and Classification

Microbial toxins are metabolites produced by plant pathogens, which play a role in host-pathogen interactions and in disease expression. They are low molecular weight substances produced by some pathogens which are capable of reproducing symptoms similar to that found in natural infections in plants (Amusa, 2006). According to Scheffer (1983), phytotoxins are a product of microbial pathogens, which should cause an obvious damage to plant tissue and must be known with some confidence to be involved in disease development. For toxic metabolites of pathogens to be regarded as phytotoxin, when applied at a concentration to the plant, the metabolites should produce in a susceptible host all the symptoms characteristic of the disease. Also the pathogen and the toxic metabolite must exhibit similar host specificity. Furthermore, the pathogen and its toxin must be able to induce similar disease symptoms and finally a single toxin must be involved. Toxins differ from enzyme in that they do not attack the structural integrity of the tissue but they affect the metabolism in a subtle manner (Amusa, 2006). Phytotoxins act directly on the protoplast of the plant cell.

Several characteristics have been used for the classification of toxins that affect plants. Such features include their chemistry. Based on this, some phytotoxins are regarded as low molecular weight peptides, others have terpenoid structures and still others contain carbohydrates. However, few other structures are known for toxins that play an unquestionable role in plant disease (Scheffer and Briggs, 1981). Another form of classification is based on the producing organism (fungi, bacteria). This is, however, of

no predictive value since more than one type of phytotoxins can be produced by one organism.

Phytotoxin classification has also been based on biological activities such as enzyme inhibitors, antimetabolites, membrane-affecting compounds (Scheffer and Briggs, 1981). However, the widely accepted classification is that based on toxin selectivity to plant genotypes (host selective or non-host selective) and on the general role in disease development (Amusa, 2006).

Species of *Colletotrichum* have been found to produce phytotoxic metabolites. Such toxic metabolites include colletotin from *Colletotrichum fuscum* Laub, colletotrichin and colletopyrone from *C. nicotianae* (Amusa, 2006). Identified metabolites from other pathogens include piricularin from *Piricularia oryzea*, victorin from *Cochliobolus vitoriae*, phaseolotoxin from *Pseudomonas syringae* pv *phaseolicola*, sacchari-toxin from *Helmithosporium sacchari* (Amusa, 2006) and cercosporin from *Cercospora* spp. (Daub, 1982). *In vitro* production of several fungal phytotoxins by *Mycosphaerella fijiensis* and *Mycosphaerella musicola* has also been reported (Svabov and Lebed 2005). *Fusarium* species produce a variety of potent phytotoxins such as fumonisins, moniliformin, fusaric acid, 2,5-anhydro-Dglucitol (AhG) and trichothecenes (Abbas *et al.*, 1991; Abbas and Boyette, 1992; Jin *et al.*, 1996; Tanaka *et al.*, 1996)

2.10.2 Microbial Toxin and Plant Disease Development

The ability of a pathogen to infect and invade a compatible host may be facilitated by the production of toxins that induce cell death in the proximity of the invading organism (Baker *et al.*, 1997; Dangl and Jones, 2001). These toxins were also reported to play important roles in inhibiting the physiological processes in cells surrounding the point of

infection, enabling the spread of the disease (Feys and Parker, 2000; Staskawicz *et al.*, 2001). It has been suggested that some pathogens would be unsuccessful if the toxin did not kill the cells in advance of the fungus and permit it to establish itself continually on dead or dying cells and produce more toxins (Amusa, 2006). While Baker *et al.*, (1997) reported that the virulence of an organism is sometimes enhanced by its ability to produce phytotoxins that kill cells in the tissue surrounding the point of infection.

In some plant diseases, especially with yam anthracnose, toxins often produced a more rapid and extensive invasion by the pathogen than would be the case in the absence of toxins. Amusa *et al.*, (1993) reported the extraction of phytotoxic metabolites from *Colletotrichum gleoesporioides* infected yam leaves. The extracted phytotoxic substance induced necrotic lesion similar to the symptoms induced by the pathogens on healthy yam leaves.

Phytotoxins often act as the initiation factor for successful pathogenesis. Spores of some fungal pathogens have been associated with phytotoxin production, which probably kills cells of susceptible host paving way for the penetration of the germ tube. All known host-specific toxins can be detected from the spore germinating fluids of each virulent pathogen but not from those of the avirulent ones (Nishimura and Kohomoto, 1983; Nutsugah *et al.*, 1994; Otani *et al.*, 1998; Quayyum *et al.*, 2003). Thus, specificity found to be characteristically associated with host specific toxin suggests the early participation of toxin at the site of initial contact of inoculated and host surface.

Several phytotoxins are now known beyond reasonable doubt, to be the determinant factor in pathogenesis and some can even act as reliable surrogates for the pathogens that produce them. Amusa (1994) reported that the partially purified metabolites of

Colletotrichum spp. induced necrotic lesion of varying sizes on leaves and stems of susceptible hosts, while the phytotoxic metabolites of *Colletotrichum graminicola, C. truncatum* and *C. lindemutianum* inhibited seed germination in respective host crops (Amusa, 1994).

One of the first physiologically detectable events induced by a phytotoxin is an increased loss of electrolytes from susceptible leaves (Kohomoto *et al.,* 1987) Nishimura and Kohomoto (1983) reported that *Alternaria kikuchiana* toxin released during spore germination on leaves caused an almost instantaneous increase in electrolyte loss from susceptible but not from resistant tissue. Different phytotoxins are known to have different modes of action. Kimura *et al.,* (1973) reported that a phytotoxin produced by *Colletotrichum lagenarium* was found to function as an anti-auxin, while sensitivity of sugarcane clones relating to electrolyte leakage caused by *Helminthosporium sacchari* toxin has been reported (Scheffer and Livingstone, 1980).

Most of the phytotoxic metabolites act by modifying the metabolism of the host plants, while some are toxic to the plant tissues once accumulated and poison the plant tissues. A phytotoxin secreted by *Psuedomonas syringae* pv. *Tabaci*, the pathogen inducing wild fire disease of tobacco, drastically modifies the amino acid metabolism of the plant with the eventual accumulation of ammonia in tobacco leaves, which causes extensive blighting. Interestingly, the pathogens that synthesize the phytotoxin remain unaffected by the toxin (Amusa, 2006).

CHAPTER THREE

3.0 MATERIALS AND METHODS

3.1 Survey for Pathogens

Field trips were undertaken to observe and examine waterways and lagoons of Badagry, Mile 2, Lagos, and Ogun River (Isheri) and to collect fungal pathogens from diseased water hyacinth. Sampling was done randomly at each of the sampling station, (Lagos Lagoon, Mile 2, Ogun river and Badagry creeks) using a motorized canoe for a period of 3 years to collect fungal agents which may colonize the plants at various seasons of the year. The coordinates of the sampling stations were also obtained with the aid of a Geographical Positioning Instrument.

3.2 Fungal Isolation from Diseased Water Hyacinth Tissue

Two mm^2 pieces were cut from the margins of necrotic lesions on the leaves. These were surface disinfected in a solution of 0.26% sodium hypochlorite for 1 min and rinsed three times with sterilized water to remove traces of the disinfectant (Martinez and Charudattan, 1998). Five leaf pieces were placed on pre-formulated potato dextrose agar (PDA), tap water agar (TWA) and carrot decoction agar (CDA), each containing antibiotics (ampicillin; 500 mg/L), in petri plates and incubated at 25°C, with a 12 hours dark/light regime to stimulate sporulation. All emerging fungi were isolated and the pure cultures were obtained by the single conidial technique (Martinez and Charudattan, 1998).

3.3 Fungal Identification

The fungal pathogens were identified according to their morphological appearance on the plates and the characteristics of spores under the light microscope. All isolates were

screened for their pathogenicity to water hyacinth by satisfying Koch's postulates (IMPECCA Technical Guide Series No. 1, 2001).

3.4 Pathogenicity Screening

Healthy water hyacinth plants were collected, washed with a 0.26% sodium hypochlorite solution and rinsed three times to eliminate insect infestation. This was maintained in the recommended growth solution: 50% Hoaglands solution (Martinez and Charudattan, 1998) and allowed to equilibrate in the solutions for one week prior to inoculation with the pathogen. The inoculum (Spore suspension) was prepared as described by the IMPECCA Technical Guide Series No. 1 (2001). Spores were harvested from PDA plates in sterile distilled water (SDW) containing 0.1% v/v Tween 80 solution. Leaves and petioles of experimental plants contained in three pots labeled A, B and C were inoculated (sprayed) with a 1x 10^6 spores/ml spore suspension using a hand held low-pressure atomizer at a distance of 20 cm from the plant until droplets form at leaf apex. A control experiment was set up by spraying leaves and petioles with sterile distilled water plus Tween 80. Inoculated plants were immediately covered with sterile polythene bags for 48 hours to maintain high relative humidity. The plants were left in the field under the conditions of average temperature ranging between 24°C and 31°C, relative humidity between 68 in the night and 86 in the day and at an average rainfall of 25 mm. The average sunlight/intensity was 7 hours per day. Plants were monitored at three day intervals for symptoms development. The isolates were ranked on the basis of the severity of the disease they inflict. Disease severity was accessed according to the IMPECCA Technical Guide Series No. 1 (2001). Finally, pathogens were re-isolated from the dead plants to confirm Koch's postulates.

3.5 Molecular Characterization of the Pathogenic Isolate

The pathogenic isolate which was tentatively identified as *Myrothecium* species was sent to the Centre for Agriculture and Bioscience International (CABI), Egham, Surrey, United Kingdom for standard and molecular identification. The molecular identification procedure was carried out as described below.

3.5.1 DNA extraction

The pathogenic isolate was subcultured onto one plate of 2% Malt Agar and two plates of Potato Dextrose Agar to produce good mycelial growth and incubated at 25°C for 2 weeks. A loopful of mycelium was scraped from one Potato Dextrose Agar plate and DNA extracted using a DNeasy Plant Mini kit (Qiagen, UK) according to the manufacturer's instructions for fungal DNA extraction. Final elution was into 100 µl sterile distilled H_2O

3.5.2 PCR amplification

The extracted DNA was subjected to PCR in a Hybaid PCR Express thermal cycler according to the following conditions:

Amplification reactions were carried out with primers ITS6 & ITS4 (Sigma Genosys, UK) in volumes of 50 µl containing 2 µl of template DNA, 1 µM of each primer (primers ITS6 & ITS4; Sigma-Genosys, UK), 200 µM of each dNTP (Amersham Pharmacia, UK), 0.05 units HotStar Taq DNA polymerase (Qiagen, UK) and 2.5 µl of 10x PCR buffer (Qiagen, UK) in sterile PCR grade H_2O. Reaction conditions: initial enzyme activation step at 95°C for 15 min, followed by 30 cycles each consisting of a denaturation step at 95°C for 1 min, an annealing step at 52°C for 1 min and extension step at 72°C for 1 min; followed by a final extension step at 72°C for 10 min after the last cycle.

3.5.3 Agarose gel electrophoresis of amplicon

Aliquots of the amplification products were subjected to electrophoresis as follows: 2 µl PCR product were added to 8 µl DNA loading buffer and loaded into a well of a 1.5% (w/v) Seakem LE agarose gel (BMA, UK). The gel was subjected to electrophoresis in half-strength tris/borate/EDTA buffer (0.5 x TBE) for 2 h at 5 Vcm^{-1}.

DNA fragments were visualised by staining with Gels as follows: fragments were stained in 0.5 mg/liter ethidium bromide solution (Sigma, UK) for 30 min and visualised on a UV trans-illuminator. Gel images were recorded using the GDS 5000 gel documentation system (UVP Ltd.) and stored as TIF bitmaps. Purity of the sample was indicated by visualisation of a single DNA band.

3.5.4 DNA sequence reaction

The remaining unused PCR products were prepared for use in sequence reactions by the application of a QIAquick PCR purification kit (Qiagen, UK), according to the manufacturer's instructions, with final elution into 30 µl sterile PCR grade H_2O.

Sequencing reactions were undertaken using BigDye v1.1 (Applied Biosystems, UK) according to the manufacturer's instructions and amplified in a Primus 96 Plus Thermal Cycler, as follows: 96°C/ 1 min followed by 25 cycles of 96°C / 20 s; 50°C/ 10 s; 60°C / 4 min. Separate sequencing reactions were undertaken for the forward (ITS6) and reverse (ITS4) primers.

Excess unincorporated BigDye was removed using DyeEx 2.0 spin kits (Qiagen, UK) according to the manufacturer's instructions. Final elution was in 10 µl sterile PCR grade H_2O. Samples were vacuum dried and resuspended in 10 µl HiDi formamide (Applied

Biosystems, UK). These resuspended samples were transferred to a 96-well plate and subjected to capillary electrophoresis sequencing in a ABI 3130 Genetic Analyser. Sequence files were obtained and these were screened against the holdings of EMBL.

3.6 Histopathology of the diseased water hyacinth.

Healthy water hyacinth leaves were infected with 1 x 10^6 spore suspension of an indigenous strain of *Myrothecium* species and allowed to stand for 7 days. A control experiment was set up by spraying another healthy water hyacinth plant with distilled water. Leaf samples were collected from both plants on a daily basis and preserved in 70% ethanol for anatomical studies. A diseased leaf from each stage, as well as a healthy leaf (of similar age) was embedded transversely in the cork and free-hand sections were cut with a sterilized razor blade. The cut sections were placed in 5% commercial bleach solution for about 15 min. after which they were transferred into distilled water for another 10 min and finally rinsed in three changes of distilled water. The cut pieces from each of the disease stage and healthy leaf were placed on a microscopic slide and stained with Lactophenol cotton blue (Adekunle *et al.*, 2005). A drop of glycerin jelly was added to the slide and then covered with a cover slip before it was mounted on the Light microscope for anatomical observations at x10, x40 and x100.

3.7 Host Range Specificity

Twenty-six indigenous and economically important agricultural crops were inoculated with the pathogenic organism; an indigenous strain of *Myrothecium* sp. according to the method of IMPECCA Technical Guide Series No. 1 (2001) as above. The crops were monitored at three day intervals for two weeks for symptoms development. The selected crops for this study included: Acalypha (*Acalypha cordifolia*), Aloe vera (*Aloe vera*),

Amarantus (*Amaranthus viridis*), Banana (*Musa sapientum*), Beans (*Vigna unguiculata*), Cabbage (*Brassica oleracea*), Carrot (*Daucus carota*), Cassava (*Manihot esculentum*), Cocoyam (*Colocasia esculentum*), Corn (*Zea mays*), Date palm (*Phoenix dactylifera*), Long-fruited Jute (*Corchorus olitorius*), Garden egg (*Solanum melongena*), Groundnut (*Arachis hypogea*), Lemon grass (*Cymbopogun citratus*), Lettuce (*Lactuca taraxacifolia*), Mango (*Magnifera indica*), Okro (*Hibiscus esculentus*), Pawpaw (*Carica papaya*), Pineapple (*Ananas comosus*), Red savina (*Capsicum chinense*), Plumed celosia (*Celosia argentea*), Chili pepper (*Capsicum annuum*), Tomato (*Lycopersicum esculentus*), Water melon (*Citrullus lanatus*), Yam (*Dioscorea alata*).

3.8 Growth Medium Formulation for Fungal Species

Fungi growth was examined on different media that have been described for fungi, this includes; Potato dextrose agar, Potato carrot agar, Carrot decoction agar, Water agar and Sabouraud agar. A semi artificial diet, which included the material from the fungal host's plant was also used in the fungi feeding trial. Fungal growth was assessed by diameter measurement on agar plate.

Preparation of Culture Media: Six culture media were used to access the mycelial growth of the fungus. The media includes: PCA medium (potatos 20 g, carrot 20 g and agar 20 g), PDA medium (diced potatos 200 g, dextrose 15 g and agar 20 g), SA medium (glucose 40 g, peptone 10 g and agar 15 g), TWA medium (agar 18 g), WHA medium (dried powdered water hyacinth leaf 50 g, agar 18 g and fresh hyacinth leaf extract {100 g/litre of distilled water}) and ZA medium (sodium nitrate 2 g, potassium nitrate 1g, potassium chloride 0.5 g, magnesium sulphate 0.5 g, ferrous sulphate 0.01 g, sucrose 30 g

and agar 20 g). Each culture medium was prepared in 1litre of distilled water and autoclaved at 120°C at 15 psi for 20 min.

Growth Measurement: The diameter growth measurement for the isolates on agar plates (5 replicates per isolate) was done according to the method of Fawole and Oso (1988). Two bisecting lines were drawn on the lower part of a sterile Petri dish. The prepared medium (10 ml) was added into the sterile Petri dish and allowed to solidify. A mycelial plug of the isolate was cut with a cork borer and placed in the centre of the Petri dish at the point of bisection. This was incubated at 25 ± 2°C. The diameter growth of the isolate was measured along the two bisecting lines. And the average diameter measurement was recorded. At the end, sixth day of the growth study, the spore concentration was estimated for each medium using a Neubaurer haemocytometer 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 onto a Neubaurer haemocytometer slide and the spores were estimated as:

Spore concentration (Spores/ml) = Number of spores counted/Number of area counted X Dilution factor X 10^6.

3.9 Fungal Growth on Different Nitrogen Sources

This was done according to the method of Fawole and Oso, 1988. The nitrogen requirement of the isolates was investigated on different enrichment media "Czapek-Dox" with its sodium nitrate substituted with equal amount (2 g/litre) of different nitrogen sources such as: ammonium chloride, ammonium nitrate, glutamine and sodium glutamate. These were also autoclaved as above. A solid state fermentation was

employed. Fungal growth was assessed by diameter measurement on agar plate as described above.

3.10 Evaluation of the Optimal pH for the Growth of the Organisms

This was carried out according to the method of Fawole and Oso (1988). A 1 ml spore suspension of the organisms was cultivated for 24 hours in various pH ranges (pH 5.5 – 8.6) of potato dextrose broth. Spores were harvested by centrifuging the broth at 3,500 g for 10 min. The pellets were washed thrice and resuspended in the same volume of water (volume of PDB). Optical density was measured at 530 nm in a UV spectrophotometer (SG8 072218, Spectronic GENESYS 8, England).

3.11 Induction & Quantification of the Cellulytic Activity of the Phytopathogens

Cellulolytic enzymes were induced in submerged cultures of the wild type phytopathogenic strains. Four mycelial plugs of 10 mm diameter cork borer were grown on the Czapeck Dox broth containing the appropriate carbon source (Carboxymethyl cellulose, Sawdust and Homogenized dry water hyacinth leaf) and incubated at 25°C for 2 weeks. At two day intervals, aliquots were centrifuged at 12,000 x g to obtain supernatant for enzyme assay. The enzyme activity was measured as micromole sugar released per milligram protein per min.

3.11.1 β-1,4-endoglucanase activity

The β-1,4-endoglucanase activity was determined by a modification of the method of Zaldivar *et al.*, (2001), using carboxymethylcellulose (substitution degree 0.7, Sigma) as substrate and the formation of reducing sugars was measured with Dinitrosalicylic acid (DNS) method. The reaction mixtures containing 10 mg CMC (Carboxymethyl cellulose) in 1 ml of 0.05M sodium acetate buffer (pH 5.0) and 1ml culture supernatant were

incubated at 50°C for 30 min. The reducing sugar formed was measured with dinitrosalicyclic acid (DNS). One milliliter (1 ml) of DNS reagent was added to 3 ml of the test sample. The colour was developed by boiling the mixture in water bath for 5 min. absorbance was read at 540 nm using spectrophotometer (SG8 072218, Spectronic GENESYS 8, England). Reducing sugar concentration was obtained from a standard glucose curve (Appendix).

3.11.2 β-1,4-exoglucanase activity

The β-1,4-exoglucanase activity was assayed as above using microcrystalline cellulose (Avicel) as substrate.

3.11.3 β-glucosidase activity

The β-glucosidase activity was assayed by incubating 0.1 ml of the culture filtrate with 0.5 ml of 0.05M acetate buffer (pH 5.0) containing 2.5 mg cellobiose at 50°C for 10 min (Zaldivar *et al.*, 2001). 10 µL of the glucose released was added to 1ml glucose oxidase peroxidase reagent (Sigma) and allowed to stand for 10 min at room temperature before reading the optical density at 546 nm. The concentration of the glucose released (mg/ml) was measured as OD sample/ OD Standard x Concentration of Standard.

3.11.4 Xylanase activity

The xylanase activity was determined by measuring the release of reducing sugars and oligosaccharides from a solution of water soluble birch wood xylan using dinitrosalicylic acid (DNS) method (Gawande and Kamat, 1999). The reaction mixtures containing 10 mg Xylan (Fluka BioChemika, 95588) in 1 ml of 0.05M sodium acetate buffer (pH 5.0) and 1 ml culture supernatant were incubated at 50°C for 30 min. The xylose formed was measured with Dinitrosalicyclic acid (DNS).

3.11.5 Total extracellular protein

The total extracellular protein was determined by Lowry's method using bovine serum albumin (BSA) as standard (Lowry *et al.*, 1951). Five milliliter (5 ml) of alkaline solution was added to the protein sample solution. This was mixed thoroughly and allowed to stand at room temperature for 10 min. Folin-Ciocalteau reagent (0.5 ml) was added and mixed. After 30 min the absorbance was read against the blank at 750 nm. This procedure was also carried out using bovine serum albumin as standard. The protein concentration in the test sample was estimated using the standard curve (Appendix).

3.12 Phytotoxin Production

3.12.1 Phytotoxin Production by the Three Isolates

The phyotoxin production ability of the isolates was investigated according to the method of Charudattan and Rao (1982). *Curvularia pallescens* Boedjin, *Fusarium solani* and an indigenous strain of *Myrothecium* species that were isolated from diseased water hyacinth in this study and had been maintained at 5°C on potato dextrose agar. For toxin production, stationary cultures were grown from mycelial transfers in potato sucrose broth in 1-liter Erlenmeyer flasks containing, respectively, 250 ml of medium per flask. The cultures were grown for 3 to 4 weeks under a 12 hours photoperiod at 25 ± 2°C. The culture fluid was filtered through eight layers of cheesecloth, then through Whatman no. 1 filter paper. The cell free culture fluid was tested for phytotoxic activity against detached water hyacinth leaves in comparison with uninoculated, filtered potato sucrose broth (as control). The toxic fluid (10 µl) was applied to adaxial leaf surfaces along a

punctured site per leaf, using six leaves per treatment. Entire leaves were incubated for 48 h over moist filter papers in Petri plates under a 12-hours photoperiod at 25 ± 2°C. Phytotoxic activity of the culture fluid was determined by measuring the diameter of necrosis or by a six-point graded scale and data transformed to give the average severity index (ASI). The average severity index (ASI) was derived from the following foliar symptoms scale: 0–5, where 0 = no symptoms, 1 = 1–10% of foliage with chlorosis and/or necrosis, 2 = 11–30%, 3 = 31–70%, 4 = 71–90%, and 5 = 91–100% (Duarte and Archer, 2003). Assays were conducted thrice or otherwise stated.

3.12.2 Effect of Light Regimes on Toxin Production

The effect of light regimes on phytotoxin production by an indigenous strain of *Myrothecium* species was investigated by the method of Duarte and Archer (2003). To obtain filtrates of the fungal cultures, flasks containing 250 ml potato-sucrose broth were inoculated with one 10-mm-diameter mycelia disk of *Myrothecium roridum*. Flasks were incubated statically up to 30 days at 25 °C under continuous illumination provided by two 40-watt fluorescent tubes at a distance of 40 cm. A duplicate experiment was set up and incubated under darkness. Filtrates were harvested by filtering first through a cheese cloth, twice through Whatman no. 1 paper before being centrifuged at 3,500 g for 15 min. Filtrates were then stored at 5 °C, in sterile bottles. Toxicity of culture filtrate was measured as average severity index (ASI).

3.12.3 Effect of pH on Toxin Production

The pH dependence on phytotoxin production by an indigenous strain of *Myrothecium* species was also investigated by the method of Duarte and Archer (2003). The effect of pH on the indigenous strain of *Myrothecium* species toxin production was studied after

growing the isolate in potato sucrose broth (PSB) culture medium with pH adjusted to 4.0, 4.5, 5, 5.5, 6, 6.5, 7 and 7.5 prior to sterilization. The pH was adjusted by adding drops of 1M NaOH or 1M CH_3COOH solution. Stationary flasks were incubated at 25 °C under continuous illumination for 25 days. Culture filtrates were tested at concentrations of 10%, 20% and 50% in a detached leaf bioassay using the water hyacinth leaf. Distilled water (pH 4.7) was used as control. Toxicity of culture filtrate was measured as ASI.

3.12.4 Effect of Different Media on Toxin Production

The media dependence of phytotoxin production by the indigenous strain of *Myrothecium* species was examined by the method described by Yoshida *et al.*, (2000). Media used for incubation of the fungus were potato sucrose broth (PSB: potato, 200g; sucrose, 20 g; distilled water, 1liter), potato dextrose broth (PDB: potato, 200 g; dextrose, 20 g; distilled water, 1liter), sabouraud broth (SB: peptone, 10 g; glucose, 40 g; distilled water, 1 liter), and Malt extract broth (MB: malt extract, 20g, distilled water, 1 liter). Potato carrot broth (PCB: potato, 20 g; carrot, 20 g; distilled water 1 liter) and Czapek Dox broth (ZDB: $NaNO_3$, 2 g; K_2HPO_4, 1 g; $MgSO_4 \cdot 7H_2O$, 0.5 g; KCl, 0.5 g; $FeSO_4$, 10 mg; sucrose, 30 g; and distilled water, 1 liter) were also used. In addition, homogenized water hyacinth leaf broth (WHB: raw water hyacinth leaf homogenized for 3 min, 145 g; and distilled water, 1 liter) was formulated. A quantity of ampicillin (250 mg per liter) was added into a 250 ml conical flask containing 50 ml each of the medium. The media were then autoclaved for 20 min at 121°C. Each flask was inoculated with a 10mm mycelial agar plug of the isolate. Stationary flasks were incubated at 25°C under continuous illumination for 21 days and liquid cultures were harvested, filtered through a cheese cloth, twice through Whatman no. 1 paper circles before being centrifuged at 3,500 g for

15 min. This was diluted to 100%, 70%, 50%, 30% and 10% concentration for biological activity assay on the water hyacinth leaf. About 6 cm^2 water hyacinth leaves were cut and punctured at the center with a needle and placed in a Petri-dish containing a moistened cotton wool. The diluted culture filtrates (10µl) were then applied on the adaxial surface of the punctured site of the leaves in separate dishes. These were incubated at 25°C under light. Each treatment was replicated six times. Toxicity of culture filtrate was measured as ASI after 48 hours of crude toxin application when symptoms were well evident.

3.12.5 Effect of Carbon Source on Toxin Production

The carbon source dependence of phytotoxin production by an indigenous strain of *Myrothecium* was examined by the method of Barbosa *et al.*, (2002). To test different defined culture media on the production of toxic metabolites, the *Myrothecium* was cultured in Czapek-Dox salts solution (ZDB: $NaNO_3$, 2 g; K_2HPO_4, 1 g; $MgSO_4 \cdot 7H_2O$, 0.5 g; KCl, 0.5 g; $FeSO_4$, 10 mg; sucrose, 30 g; and distilled water, 1 liter) with the sucrose substituted with equivalent amount of sugar types (fructose, glucose, xylose, maltose, galactose and lactose). The media were autoclaved, inoculated and incubated as in the experiment above. Toxicity of culture filtrate was measured based on the diameter of necrosis after 48 h of crude toxin application when symptoms were well evident.

3.12.6 Effect of Nitrogen Source on Toxin Production

The nitrogen source dependence of phytotoxin production by the *Myrothecium* species was also determined by a slight modification of the method of Barbosa *et al.*, (2002). To test different defined culture media on the production of toxic metabolites, the *Myrothecium* was cultured in Czapek-Dox salts solution (ZDB: $NaNO_3$, 2 g; K_2HPO_4, 1 g; $MgSO_4 \cdot 7H_2O$, 0.5 g; KCl, 0.5 g; $FeSO_4$, 10 mg; sucrose, 30 g; and distilled water, 1

liter) with the sodium nitrate ($NaNO_3$) substituted with equivalent amount of exogenous nitrogen containing salts (NH_4NO_3, NH_4Cl, Na-glutamate, Glutamine, Yeast extract, and Urea). The media were autoclaved, inoculated and incubated as in the experiment above. Toxicity of culture filtrate was measured based on the diameter of necrosis after 48 h of crude toxin application when symptoms were well evident.

3.13 Biological Activity of Toxin

3.13.1 Effect of Light on Phytotoxin Activity

To determine the effect of photoperiod on biological activity of the crude fungal toxin on water hyacinth, about 6 cm^2 leaves were cut and punctured at the center with a needle and placed in a Petri-dish containing a moistened cotton wool. Diluted culture filtrate (10 µl) of the non-autoclaved lyophilized toxin powder at concentrations (w/v) of 2%, 1%, 0.5%, 0.25%, 0.02% and 0.01% were then applied on the adaxial surface of the punctured site of the leaves. These were incubated at 25°C under light; a second set of the experiment was incubated in darkness simultaneously. Each experimental set up was replicated six times. Toxicity of culture filtrate was measured as ASI after 48 hours of crude toxin application when symptoms were well evident.

3.13.2 Effect of pH on Phytotoxin Activity

The culture filtrate was diluted (to 100%, 50% and 10% concentration) in buffer (KH_2PO_4 and K_2HPO_4 buffers of pH 4 – 7.5) to examine the effect of pH on phytotoxin activity. Freshly made buffered solutions containing the phytotoxin were assayed for biological activity and scored as outlined previously. Appropriate solutions consisting of only buffer at each pH were diluted with water to 100%, 50% and 10% concentration and applied topically to water hyacinth leaves as controls and were assessed and scored in the

same way as the culture filtrate. Positive results from controls were deducted from the tests screening phytotoxin activity to correct for buffer interference. Toxicity was expressed as average severity index (ASI) as above.

3.13.3 Effect of Heat on Biological Activity of Crude Toxin

In order to determine the effect of autoclaving culture filtrate on symptom development on Water hyacinth, about 6 cm^2 leaves were cut and punctured at the center with a needle and placed in a Petri-dish containing a moistened cotton wool. A diluted culture filtrate (10 µl) of both autoclaved (121°C for 20 min) and non-autoclaved phytotoxin containing media at concentrations of 100%, 50% and 10% were then applied on the adaxial surface of the punctured site of the leaves in separate dishes. These were incubated at 25°C under light and each treatment, replicated six times. Toxicity was measured as ASI after 48 hours of crude toxin application when symptoms were well evident.

3.14 Biological Activity of Phytotoxin on Leaves of Various Plants.

Healthy leaves from 26 species of randomly selected plants (as in *M. roridum* host range specificity experiment) cultivated in the field were sampled and cut into 6 cm^2 fragments with scissors. Culture filtrate (10 µl) obtained from an indigenous strain of *Myrothecium* species was then placed on the adaxial surface of the leaves punctured with needles. After incubation for 2 to 3 days in a moist petri-dish at room temperature, toxicity of the extract was determined by the appearance of necrotic lesions on the leaves. Toxicity was measured as ASI.

3.15 Isolation and Partial Characterization of Phytotoxin Produced by an Indigenous Strain of *Myrothecium* Species

3.15.1 Fungal Source, Culture and Toxin Extraction

The fungus; an indigenous strain of *Myrothecium* species was isolated from diseased water hyacinth in this study and has been maintained at 5°C on malt extract agar tubes. For toxin production, stationary cultures were grown from mycelial transfers in potato sucrose broth in five 6-liter Erlenmeyer flasks containing respectively, 1 liter of medium per flask. The cultures were grown for 4 weeks under a 12 hours photoperiod at 25 ± 2°C. The culture fluid was filtered through eight layers of cheesecloth and centrifuged at 3,500 g for 15 min. The filtrate was passed through Whatman no. 1 filter paper. The filtrate was lyophilized to dry powder and stored in a sterile container before use. The filtrate was extracted thrice with 100 ml $CHCl_3$: MeOH (1:1). The extracts were pulled together and concentrated with the aid of a rotary evaporator. A small amount of the residue was redissolved in methanol and spotted onto a 0.25 mm thick precoated silica gel TLC plates (Sigma Co. United Kingdom). The developing solvent systems were EtOAc: MeOH (9:1) and EtOAc: MeOH: Water: Acetic acid (10:1.7:1.3:0.2), and the fungal metabolites were detected using short wavelength UV light at 366 nm (model: 80286, Desaga, Heidelberg, Germany) and vanillin spray (Bean *et al.*, 1984).

3.15.2 Separation, Isolation and Partial Characterization of Toxins

The crude extract was subjected to chromatography (20 g of 2-25 μ silica gel) under vacuum (flash chromatography), eluted first with 30 ml of CH_2Cl_2 followed by 50 ml of 10% MeOH in CH_2Cl_2 and finally by 50ml of 20% MeOH in CH_2Cl_2 (Bean *et al.*, 1984). The three fractions were concentrated to dryness. These were spotted differently on a

0.25 mm thick precoated analytical grade silica gel thin layer plate (Sigma Co. United Kingdom) and developed in different solvent systems EtOAc: MeOH: Water: Acetic acid (10:1.7:1.3:0.2), MeOH:CH_2C1_2 (8:92) and MeOH:CH_2C1_2:Hexane (4:92:4). Bands were detected using short wavelength UV light (model: 80286, Desaga, Heidelberg, Germany) and vanillin spray. The best resolving solvent for the toxin was used for the isolation of the various fractions on a preparative TLC plate (1 mm thick 2-25μ silica gel). The various fractions were tested for biological activity on water hyacinth leaves. The phytotoxic fraction was again subjected to an analytical grade silica gel thin layer chromatography plate for purity check.

3.16 Data Analysis

The experimental assays were done in six replicates, unless otherwise stated. Data were given to 2 decimal places and were reported as mean ± standard error of mean (SEM). Significant difference between time points in the growth studies of the isolates were determined by Fisher's protected least significant difference *t*-test with two tail probabilities of less than 0.05 considered significant. Significant differences between two experimental variations on the same isolates were assessed by a one-way analysis of variance and the Student's t-Test. These statistical analyses were done using SPSS. 11 for windows.

CHAPTER FOUR

4.0 RESULTS

4.1 Survey for Pathogens

The initial cursory observation on the field of water hyacinth in its exotic range was that there was little visible evidence to signify the occurrence of fungal pathogens. However, patches of dying plants in response to both abiotic and biotic factors were often seen. Two of the four different sampling stations; Badagry Creeks (N6.41950° E2.86019°, N6.42066° E2.86630° and N6.41748° E2.87552°) and Ogun River (N6.64091° E3.8406°) (Figure 4.1) revealed that there was a fungal pathogen present on the water hyacinth between September and November and this pathogen showed a unique pattern of infection (Figure 4.2). This pattern appeared as a leaf spot (up to 1 × 5 cm), rounded on the side facing the petiole and tapering to a narrow point in the direction of the laminar tip. Older leaf spots turn necrotic with dark brown margins, with the centre of the spot covered with discrete white and black conidial masses (Figure 4.2). The pathogenic strain caused an easy-to-identify brownish necrotic blight on the leaf, forming massive brownish patches on the leaves of the host plant in its natural habitat. The continuous survey of the water bodies over the period of investigation showed that the plant appears on the water bodies at varying seasons of the year, particularly in the rainy period between late May and late September. The plant vanishes at the dry season, the period when the salinity of the water bodies is at the maximum.

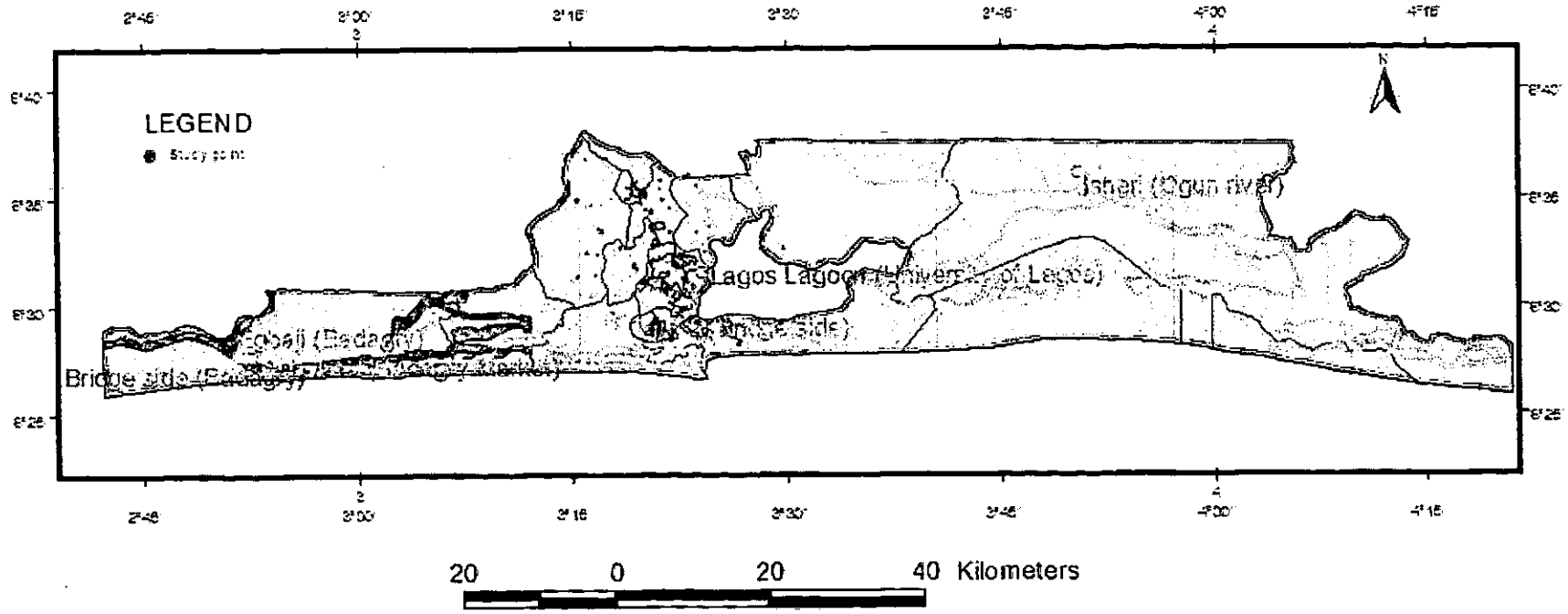

Figure 4.1: The Lagoons and Creeks of Lagos and its Environs Surveyed for Diseased Water hyacinth.

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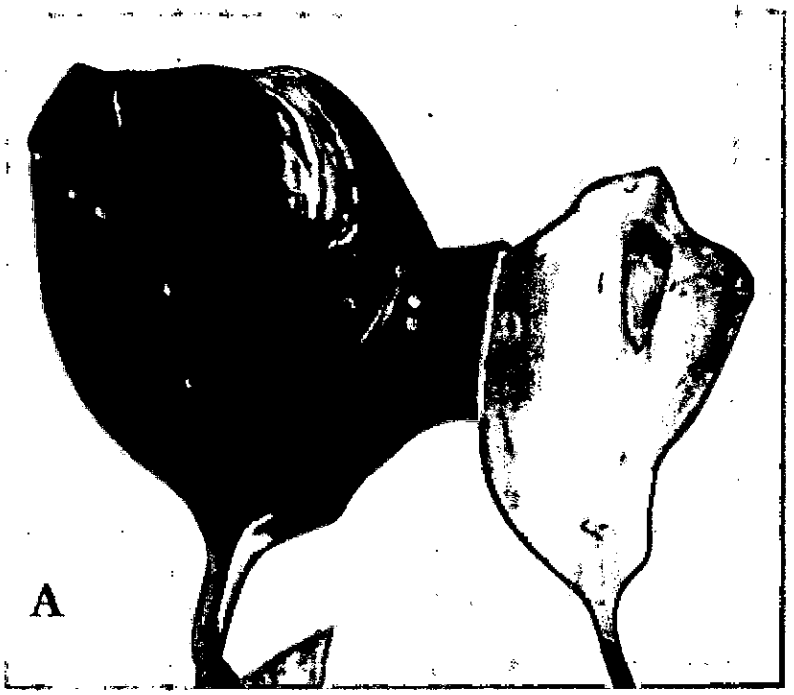

Figure 4.2: Pattern of Infection of Water hyacinth by *Myrothecium* Species on the Survey site.

4.2 Fungal Isolation from Diseased Water Hyacinth Tissue

The plant tissue sterilized with 0.26% sodium hypochlorite solution produced at least five different fungi (*Fusarium* sp., *Aspergillus niger., Aspergillus flavus, Curvularia* sp., *Penicillium* sp.) on potato dextrose agar within 24 hours and *Myrothecium* sp. within 36 hours. When the plant tissue was sterilized with 1.4% sodium hypochlorite solution *Myrothecium* sp. appeared conspicuously on day 3, while the other pathogens appeared between days 5 and 6. Of these organisms in the series of plant tissue culture made, *Myrothecium* sp. occurred most frequently (45%) followed by *Fusarium* sp (30%), *Curvularia* sp. (14.7%), *Penicillium* sp. (5%), *Aspergillus niger* (3.5%) and *Aspergillus flavus* (1.8%). The use of tap water agar (TWA) medium yielded *Myrothecium* sp. after the 24th hour, although this appeared as transparent hyphal structures on the medium as compared to its fluffy whitish conspicuous appearance on potato dextrose agar. The growth of other organisms such as *Fusarium* sp. and *Curvularia* sp. were not noticeable until the fourth to fifth day. The growth of the other organisms appeared not to be well supported by TWA.

4.3 Fungal Identification

Cultures of one of the isolates appeared with yellow to lime-green colonies on PDA plate. The hyphae are septate and hyaline. Conidiophores are non-septate. Conidial heads are radiate to loosely columnar. This was identified as *Aspergillus flavus*. The second isolate initially appeared white on PDA plate and quickly turned to black colour with dusty colonies. The hyphae are septate and hyaline. Conidiophores are non-septate. The conidial heads are mop-like, and this fungus was identified as *Aspergillus niger*. The third isolate appeared white to blue-green with flaky colonies on PDA plate. The hyphae

are septate and hyaline. The conidiophores are branched with brush-like conidial heads. The isolate was identified as *Penicillium chrysogenum*. The Cultures of the fourth isolate on PDA (using an 8mm diameter cork borer) reached 56mm diameter in 6 days at 25°C, brownish-black with irregular border and concentric zones with exudates produced (Figure 4.3A). Conidia were slightly curved, septate and the central cells were broader than end cells (Figure 4.3B). The isolate was identified and authenticated as: *Curvularia pallescens* Boedijn by Dr. Markus N. Thormann (Northern Forestry Centre, Natural Resources Canada, 5320-12251, Edmonton, AB, T6H 3S5 Canada).

Cultures of the fifth isolate on PDA reached 77.5 mm diameter in 6 days at 25°C, slightly whitish at first and later pinkish in colour. Conidia were sickled and septate (Figure 4.4). The isolate was authenticated as *Fusarium solani* by Dr. A.A. Adekunle (Botany and Microbiology Department, University of Lagos). The sixth isolate on PDA reached 77.13 mm diameter in 14 days at 25°C, isolations from lesions on potato dextrose agar produced white, floccose colonies with sporodochia in dark green-to-black concentric rings (Figure 4.5). Conidia were hyaline and cylindrical with rounded ends and averaged 7.4 × 2.0 µm. All characteristics were consistent with the description of *Myrothecium roridum* Tode ex Fr. (IMPECCA Technical Guide Series No. 1, 2001). This was authenticated as *Myrothecium roridum* Tode and was given the accession number (IMI 394934) by Dr P.F. Cannon & Dr A.G. Buddie at the Centre for Agriculture and Bioscience International (CABI), Egham Surrey, United Kingdom.

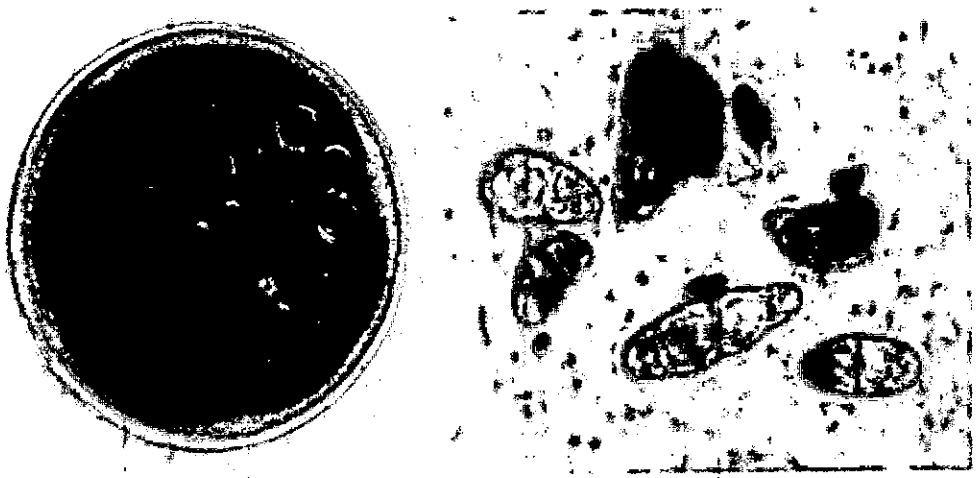

Figure 4.3: (A) Morphological Appearance of *Curvularia pallescens* Boedjin on PDA Plate. (B) Photomicrograph *Curvularia pallescens* Boedjin x1000

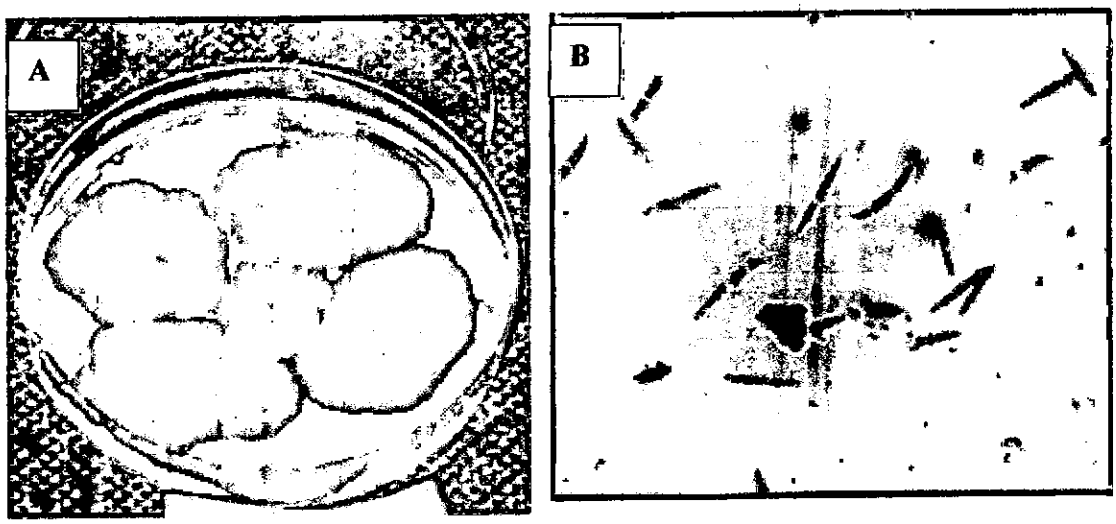

Figure 4.4: (A) Morphological Appearance of *Fusarium solani* on PDA Plate. & (B) Photomicrograph *Fusarium solani* x 1000

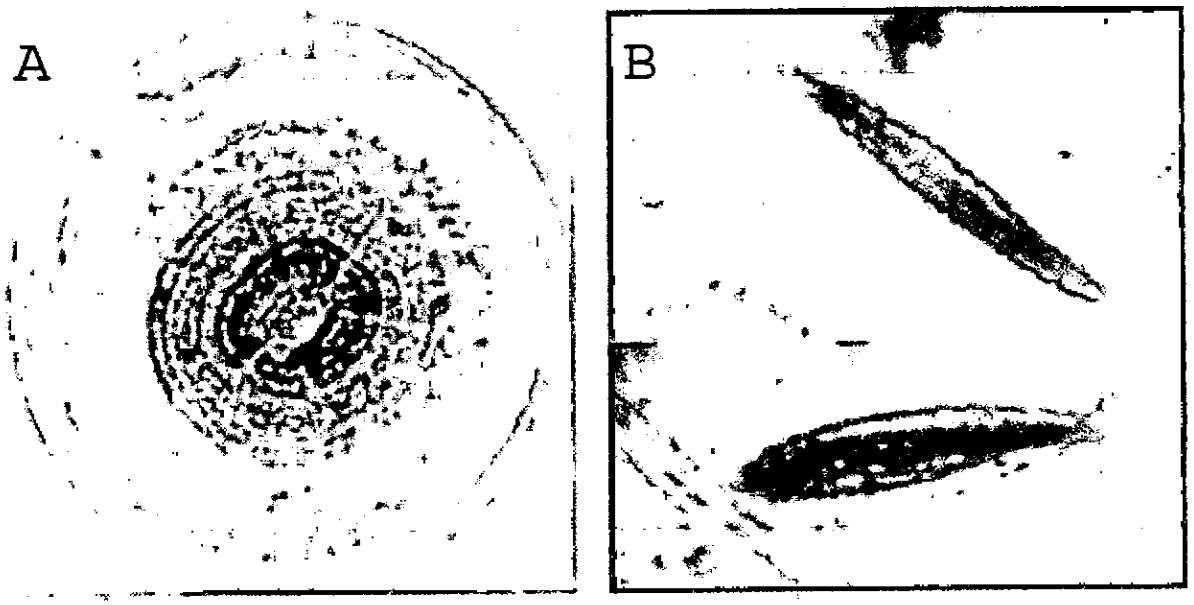

Figure 4.5: (A) Morphological Appearance of *Myrothecium roridum* (IMI 394934) on PDA Plate. & (B) Photomicrograph of *Myrothecium roridum* (IMI 394934) x 1000

4.4 Pathogenicity Screening

Prior to pathogenicity trial, healthy plants were seen to survive in tap water as against the aquatic plant culture medium (Hoaglands solution) recommended in literature. No disease symptoms were observed on the healthy plant infected with *Curvularia pallescens* Boedijn ($1x10^6$ spores/ml) and *F. solani* ($1x10^6$ spores/ml) 24 days post inoculation. Of the six different fungal species tested for their ability to infect the healthy water hyacinth plant *in vitro*, the result showed that *M. roridum* (IMI 394934) was the only candidate which was able to infect the plant and produce disease symptoms on the water hyacinth leaf. The disease started as small necrotic spots/patches and developed into pale-to-dark brown, circular leaf spots 5 to 10 mm in diameter with concentric rings observed on water hyacinth leaf. This fungus caused teardrop-shaped leaf spots, rounded on the side towards the petiole and tapering to a narrow point in the direction of the laminar tip. The older leaf spots appeared necrotic with a dark brown margin at the centre of the spot (Figure 4.6) and the leaf spots later coalesce. This isolate was ranked on the basis of the severity of the damage it caused (Table 4.1). The progression of the disease severity and disease incidence was monitored over a time interval (Figure 4.6 & Figure 4.7). The results obtained from the three sets of experiments is presented in the table below (Table 4.1). The disease incidence on day 4 was 83.33% in pot A (experiment 1) and 66.67% in pots B and C respectively. This reached 100% in all the three experiments (pot A, B and C) on day 7. Similarly, the average disease severity became prominent on day 4 in all the pots. This was 2.60 ± 0.81 in pot A, 2.75 ± 0.85 in pot B and 2.75 ± 1.60 in pot C. The average disease severity on day 24 was maximum; 8.67 ± 0.33 in pot A, 7.17 ± 1.14 in pot B and 7.17 ± 0.50 in pot C. The tagged inoculated plants in the three different pots were finally observed with heavily coalesced spots on the leaf with dead

lamina. Petioles latter submerged as a result of the absence of photosynthetic activity in the necrotic or dried leaves. The disease severity observed in the three pots were not significantly different ($P > 0.05$) when subjected to the student *t*-TEST and Analysis of variance. This is an indication that the result is reproducible.

Thus, owing to the destructive nature of this isolate; *Myrothecium roridum* (IMI 394934), it was chosen for further study in comparism with *Curvularia pallescence Boedjin* and *Fusarium solani* which have been implicated in literature as mycobiota recorded on water hyacinth (*Eichhornia crassipes*), worldwide (Barreto and Evans, 1996). The pathogenic isolate was rated highly virulent and aggressive and therefore capable of being a biocontrol agent for the water hyacinth plant. Other isolates such as *Aspergillus niger*, *Aspergillus flavus* and *Penicillium chrysogenum* which also showed no symptom in the healthy plant were excluded from subsequent studies.

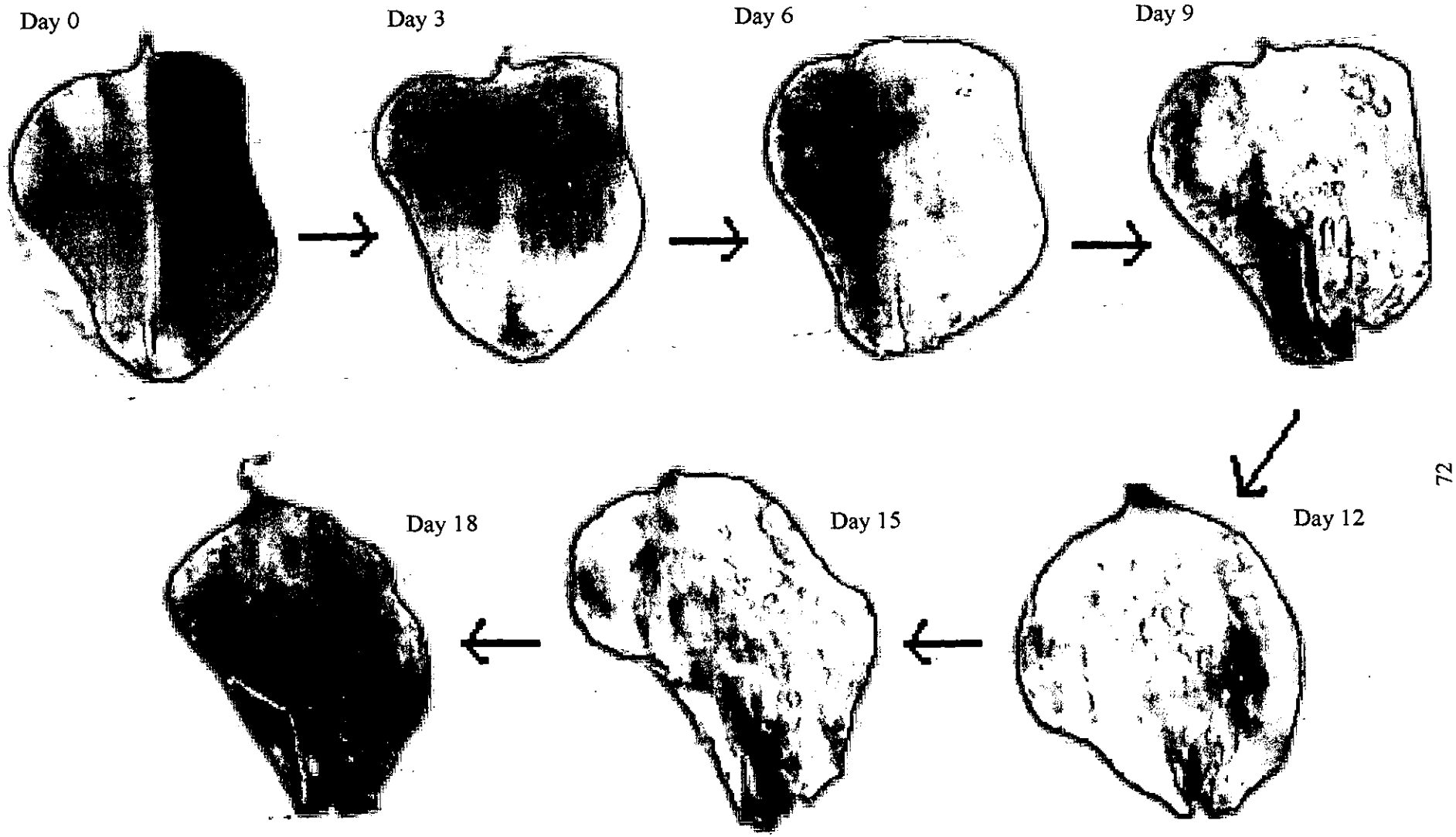

Figure 4.6: Disease Progression in Water Hyacinth Leaf Post Inoculation with *Myrothecium roridum* (IMI 394934).

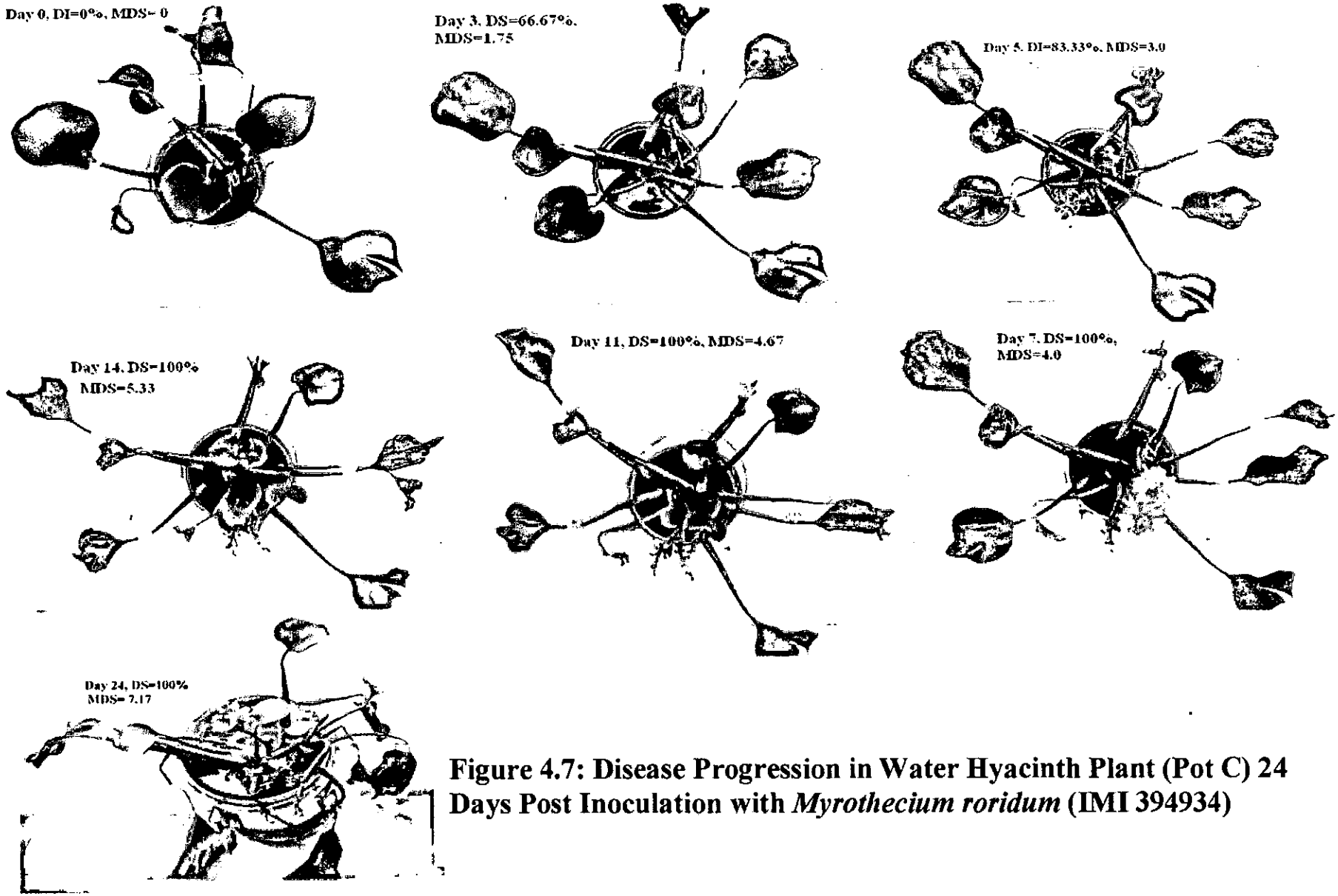

Figure 4.7: Disease Progression in Water Hyacinth Plant (Pot C) 24 Days Post Inoculation with *Myrothecium roridum* (IMI 394934)

Table 4.1. Pathogenicity Profile of *Myrothecium roridum* (IMI 394934) on Water Hyacinth.

Dates	DPI	Disease Incidence Pot A	Mean Disease severity Pot A	TTEST Pot A/B	Disease Incidence Pot B	Mean Disease severity Pot B	TTEST Pot B/C	Disease Incidence Pot C	Mean Disease severity Pot C	TTEST Pot C/A
29/11/06	0	0.00	0.00 ± 0.00	1.00	100	0.00 ± 0.00	1.00	0.00	0.00 ± 0.00	1.00
2/12/06	3	0.00	0.00 ± 0.00	1.00	100	0.00 ± 0.00	1.00	0.00	0.00 ± 0.00	1.00
3/12/06	4	83.33	2.60 ± 0.81	0.90	66.67	2.75 ± 0.85	0.79	66.67	2.75 ± 1.60	0.71
4/12/06	5	100	3.17 ± 0.79	0.89	83.33	3.00 ± 0.89	0.69	83.33	3.00 ± 1.17	0.76
6/12/06	7	100	4.17 ± 0.79	0.89	100	4.00 ± 0.86	0.80	100	4.00 ± 0.99	0.70
9/12/06	10	100	5.00 ± 0.73	0.78	100	4.67 ± 0.92	0.70	100	4.67 ± 0.83	0.88
10/12/06	11	100	5.17 ± 0.75	0.68	100	4.67 ± 0.92	0.70	100	4.67 ± 0.83	1.00
13/12/06	14	100	5.83 ± 0.75	0.70	100	5.33 ± 1.02	0.63	100	5.33 ± 0.86	0.87
16/12/06	17	100	6.83 ± 0.40	0.41	100	5.83 ± 1.08	0.38	100	5.83 ± 0.68	0.84
19/12/06	20	100	7.83 ± 0.40	0.39	100	6.67 ± 1.23	0.37	100	6.67 ± 0.68	0.84
23/12/06	24	100	8.67 ± 0.33	0.24	100	7.17 ± 1.14	0.31	100	7.17 ± 0.50	0.79

Disease Severity Keys:

0 = no spots on lamina or petiole
1 = 1 to 4 spots on lamina, no petiolar spotting
2 = Less than 25 percent of lamina surface with spots, no coalescence or petiolar spotting
3 = Less than 50 percent of laminar surface with spots, some coalescence, no petiolar spotting
4 = Less than 50 percent of leaf surface with spots, coalescence, some tip dieback, and petiolar spots
5 = Less than 50 percent of leaf surface with spots, coalescence, 10 percent tip dieback, and petiolar spotting
6 = Less than 75 percent spots, coalescence, 30 percent tip dieback, and petiolar spotting
7 = Greater than 75 percent spots, coalescence, 60 percent tip dieback, coaleascing spots on petiole
8 = dead lamina, petiole green, but heavily spotted; and
9 = dead lamina and petiole (submerged)

4.5. Molecular Identification of isolate

The report of Dr P.F. Cannon & Dr A.G. Buddie of CABI microbial identification service showed that the pathogenic isolate in this study; *M. roridum* (IMI 394934) is a common species on a wide range of plant substrata. It often occurs as a saprobe, but may cause necrotic lesions on leaves or stems. It is not known as a hazardous species in environmental or clinical situations. Morphological analysis shows that the strain conforms in all respects to standard descriptions of this species. Molecular analysis of the ITS rDNA shows >98% homology to authenticated sequences of *M. roridum*, and 99% homology to a strain identified as the closely related species *M. carmichaelii*. The genus Myrothecium has not been studied in detail using molecular phylogenetic techniques, and the difference in homology is not sufficient to establish an unequivocal identification. It is possible that the two species should be considered as synonyms. This isolate has been registered in the International Mycological Institute (IMI) Culture Collection Center.

Molecular sequence data:

[N.B. The identifications obtained via sequence homology with GenBank and/or EMBL are reliant on the validity of the identifications associated with those holdings. It should also be appreciated that there are considerable gaps in the taxa covered in these holdings].

IMI394934 ITS4

GGGTATTCCTACCTGATCCGAGGTCACTTTCGAAGTGGGGTGTTTTA
CGGCATGGCCACCGCCGAGCTCCAATGCGAGTTGTGCTACTACGCAG
AGGGGGACTACAGCGAGACCGCCACTGAATTTCGGGGCCGGCAGGC
GCGGGGACGTCCCGGAGGACGCCCCGGCCCACGCCGATCCCCAACG
CCAGGCACTGGGGGCCTGAGGGTTGAAATGACGCTCGAACAGGCATG
CCCGCCAGAATACTGGCGGGCGCAATGTGCGTTCAAAGATTCGATGA
TTCACTGAATTCTGCAATTCACATTACTTATCGCATTTCGCTGCGTTC
TTCATCGATGCCAGAACCAAGAGATCCGTTGTTGAAAGTTTTTATTTA
TTTGTTTATGTGTCACTCAGAGGAGAAAACCACTAAAGACATAAGAGT
TTGGGTTCTCCGGCGGGCGCCTGGTTCCGTTGCCCGAAGGCGCCGGG

4.6 Histopathology of diseased water hyacinth plant

The transverse section of leaf showed that the mesophyll is differentiated into a palisade and spongy mesophyll (Figure 4.8). Palisade layer is present on both upper and lower side beneath the epidermis. The upper epidermis has 5~7 layers of cells and the lower epidermis has 2~3 layers. Inside the palisade layer are densely staining material which may be supportive in nature (Figure 4.8). The spongy mesophyll consists of a large number of air spaces surrounded by thin walls full of chloroplast. Sclereids are observed in cells facing air spaces. Vascular bundles are of two types, i.e. smaller and larger vascular bundles. Smaller vascular bundles are present in both upper and lower epidermis side; some of them are in contact with the epidermis. Each vascular bundle is collateral with xylem towards the lower epidermis side and phloem towards the upper epidermis side. Tracheary elements consist of tracheids, vessels, and parenchyma cells. Tracheary elements in smaller bundles are thin-walled and without usual secondary thickenings. The phloem consists of sieve tubes and companion cells. Bundle sheath extensions are also observable in smaller bundles (Figure 4.8). Large vascular bundles are present in the leaf center and extend from one end to the other of the leaf (Figure 4.8). Each vascular bundle is surrounded by a bundle sheath of parenchyma cells. Sclereids are observable in the palisade cells, and also in air spaces. Some bundles have cross connections.

The fungus caused anatomical changes in the infected leaves. In the infected *E. crassipes* leaves (fourth day post inoculation), the cells were darkened and shrivelled, and there was loses of cell shape (Figure 4.9a-4.9c). The anatomical features exhibited in the infected leaf included a reduction in the size and number of vascular bundles, reduction in the xylem and phloem vessel, collapse of the air chambers and also the disintegration

of the palisade mesophyll (Figure 4.9a-4.9c). There was a collapse of the upper and lower palisade. There was no cuticular damage at the early stage of infection. However after six days post-inoculation, cuticular damage resulted at the later stage with subsequent cell disintegration (Figure 4.9d).

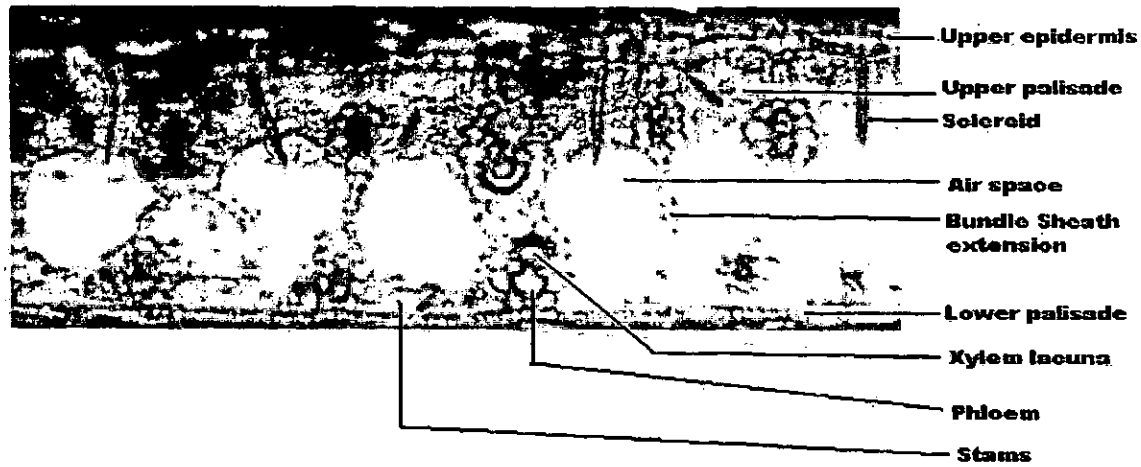

Figure 4.8: Transverse Section of Leaf of Water Hyacinth Control Plant (x40)

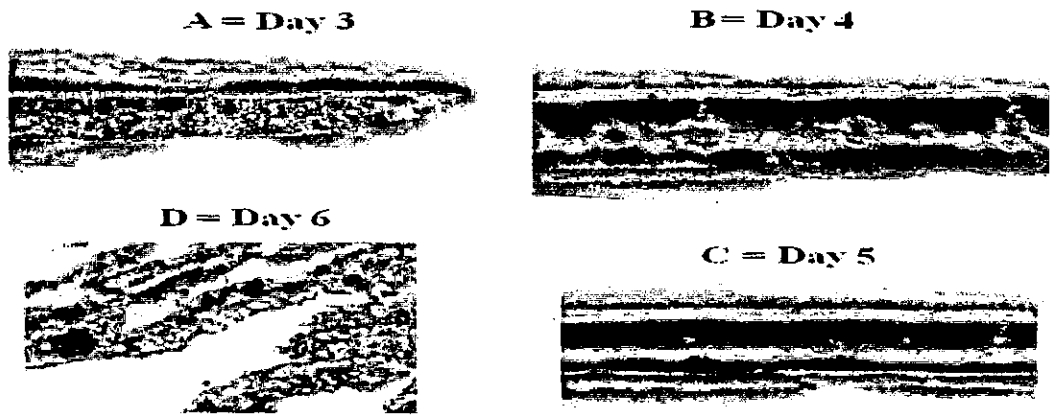

Figure 4.9: Transverse Section of Diseased Leaf of Water Hyacinth Experimental Plant with Different Degree of Anatomical Damage (x40)

4.7 Host specificity

Twenty-six indigenous and economically important agricultural crops were selected for the host range test. However; none of these crops was susceptible to the strain of *Myrothecium roridum* (IMI 394934) isolated in this study.

4.8 Effect of Media Types on Growth

A graph (Figure 4.10) of mean colony size of *C. pallescens* versus time was generated. The microbial growth increased significantly from day zero over the growth period in the media types ($P < 0.001$). The maximum growth was observed in water hyacinth agar medium (WHA) on day 6. Hence, the growth rate and final colony size on WHA were compared with the other media types using a one-way analysis of variance. A statistically significant difference in the growth rate only existed with tap water agar medium (TWA) ($P < 0.05$). Also a significant difference ($P < 0.001$) in the final colony size was observed with other media types. This is an indication that media types affect the colony size (i.e mycelial growth) of *C. pallescens*. A sharp increase in the growth was observed between day 2 and 3 in WHA medium. This suggests that day 3 signals the beginning of the exponential growth phase of *C. pallescens* and maximum utilization of the substrate. Similar drastic increase in the mycelial growth was observed with other media types between the 3rd and 4th day except Czapek Dox agar medium, which showed such increase between day 4 and 5. The maximum growth (mm) was least, 37.30 ± 0.42 with TWA and highest, 84.95 ± 0.05 with WHA on day 6.

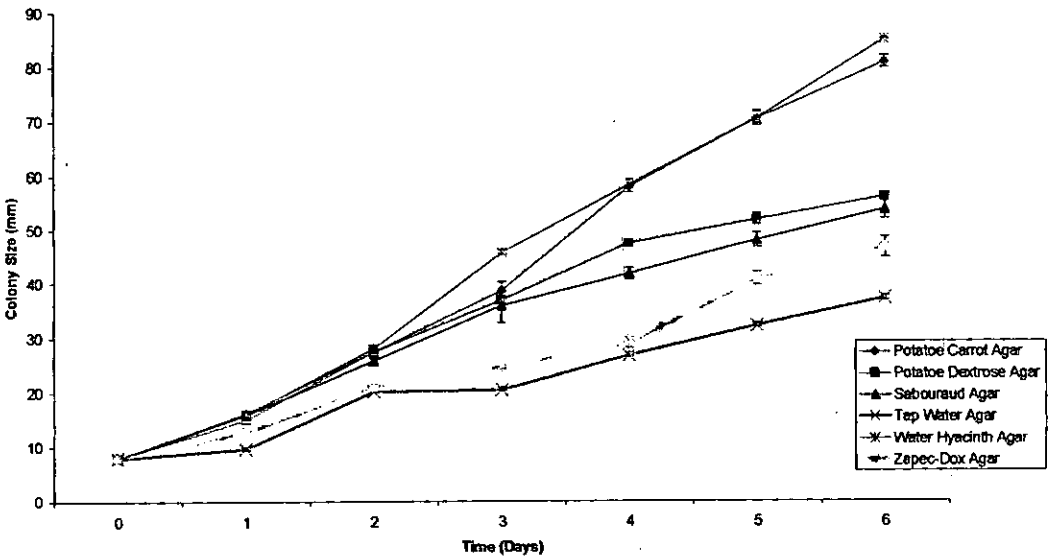

Figure 4.10: Growth Profile of *Curvularia pallescens* Boedijn on Different Types of Media. Values are

Moreso, there was a strong positive linear relationship between the time (days) and the growth of *F. solani* in all the media types; PCA, PDA, SA, TWA, WHA and Czapek Dox respectively. The microbial growth increased significantly from day zero over the growth period in the media types ($P < 0.001$). The maximum growth was also observed in water hyacinth agar medium (WHA) on day 6 (Figure 4.11). The maximum growth (mm) was least, 38.10 ± 2.79 with SA and highest, 85.00 ± 0.00 with WHA on day 6.

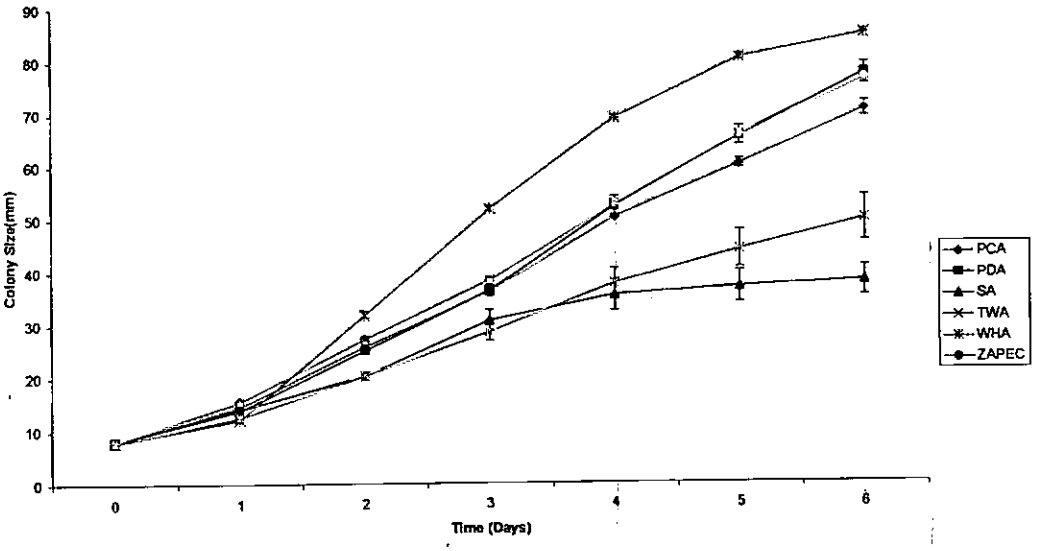

Figure 4.11: Growth Profile of *Fusarium solani* on Different Types of Media. Values are mean ± SEM for five replicate results.

Also, the same positive linear relationship existed between the time (days) and the growth of *Myrothecium roridum* (IMI 394934) in all the media types; PCA, PDA, SA, TWA, WHA, ZA, MEA and PSA. All these media supported the growth of the organism at varying degree (Figure 4.12).

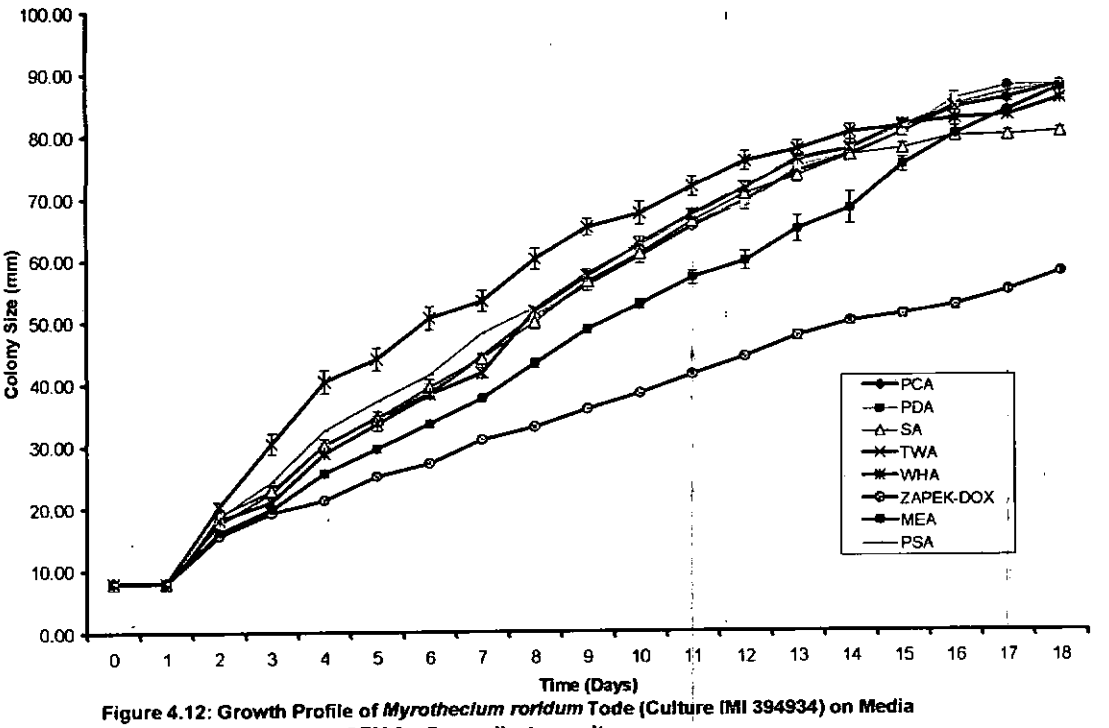

Figure 4.12: Growth Profile of *Myrothecium roridum* Tode (Culture IMI 394934) on Media Types. Values are mean ± SEM for five replicate results.

The maximum growth was observed on TWA on day 14. The growth was maximum; 80.30 ± 1.23 mm on TWA and minimum; 49.83 ± 0.60 mm on ZA (Figure 4.12). The growth rate of the organisms was determined on all the media types as colony size per day. The result obtained showed that each organism had a different growth rate on each medium (Table 4.2). The growth rate (mm/day) for *C. pallescence* was highest; 13.75 ± 1.29 mm/day on PCA and least; 5.49 ± 0.02 on TWA. The growth rate on WHA (13.70 ± 0.23) was significantly greater ($P < 0.05$) than that observed on other media types except PCA (Table 4.2). The growth rate of *F. solani* was highest on WHA and this was significantly greater than that observed on the other media types ($P < 0.05$) (Table 4.2). The maximum growth rate; 13.13 ± 3.55 mm/day of *M. roridum* (IMI 394934) observed on TWA was significantly greater than that obtained in all the media types ($P < 0.05$) (Table 4.2).

Table 4.2. Average Growth Rate (mm/day) of Isolates on Media Types

Media Types	**Isolates**		
	C. pallescence Boedjin	*F. solani*	*M. roridum* (IMI 394934)
PCA	**13.75 ± 1.29**	11.41 ± 0.01**	4.69 ± 0.13*
PDA	7.55 ± 0.63**	**13.09 ± 0.22***	4.82 ± 0.21*
SA	8.11 ± 1.47*	5.19 ± 1.72**	4.40 ± 0.41*
TWA	5.49 ± 0.02***	7.46 ± 0.35***	**13.13 ± 3.55**
WHA	**13.70 ± 0.23**	**15.94 ± 0.74**	4.81 ± 0.50*
ZA	6.95 ± 0.98**	12.08 ± 0.34**	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 for each organism using the student TTEST and ANOVA, where *$P < 0.05$, **$P < 0.01$ and ***$P < 0.001$

The spore concentration of *C. pallescens* in WHA plate as obtained with the aid of the neubaurer chamber was not appreciable (Figure 4.13). The microbial spore concentration (spores/ml) was highest, $6.67E^{+06} \pm 3.33E^{+05}$ with tap water agar medium (TWA); higher, $3.5E^{+06} \pm 5.0E^{+05}$ with PDA; high, $2.00E^{+06} \pm 0.00$ with Czapek Dox, and low, $1.00E^{+06} \pm 0.00$ with SA. The spore concentration (spores/ml) of *F. solani* was highest, $9.79E^{+08} \pm 2.32E^{+07}$ with PDA and least, $1.98E^{+06} \pm 2.25E^{+05}$ with SA (Figure 4.13). While, *M. roridum* sporulated best on malt extract agar (MEA) with an average spore concentration 29.89×10^9 spores/ml. The second best medium for sporulation was potato sucrose agar (PSA), 32.22×10^8 spores/ml and the least was observed on tap water agar (TWA). The spore concentration of this isolate on MEA was significantly greater than those of other media types used ($P<0.001$).

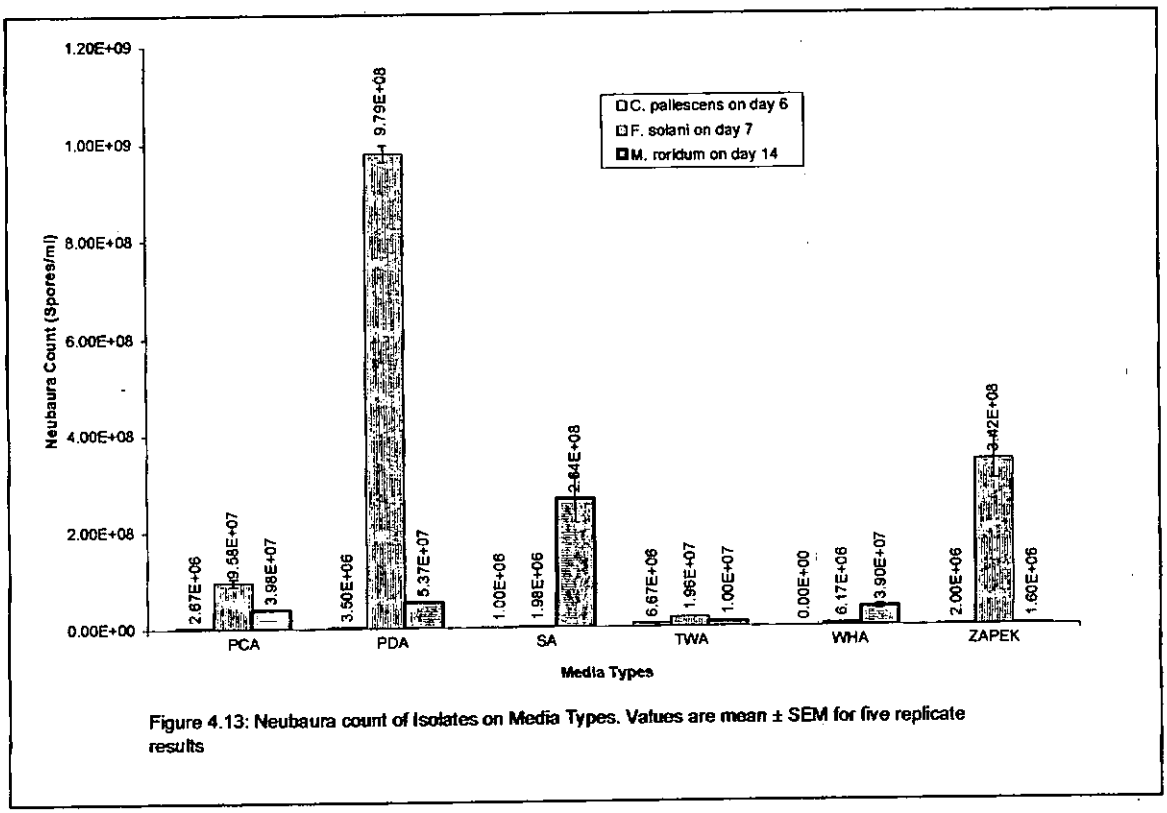

Figure 4.13: Neubaura count of Isolates on Media Types. Values are mean ± SEM for five replicate results

4.9 Effect of Nitrogen Sources on Growth

The fungus; *C. pallescens* increased in colony size in the nitrogen sources; ammonium chloride, ammonium nitrate, glutamine, sodium glutamate and sodium. Moreover, the microbial growth in the nitrogen sources increased significantly over the growth period as compared to day zero ($P < 0.01$). The maximum mycelial growth of *C. pallescens* was observed on the 10^{th} day in sodium glutamate medium (Figure 4.14). The microbial growth rate on sodium glutamate medium was compared to the other media types using a one-way analysis of variance. The growth rate on sodium glutamate was significantly different ($P < 0.001$) from that in ammonium chloride and ammonium nitrate. A significant difference ($P < 0.01$) was also observed when compared to growth rate in glutamine medium. There was no significant difference ($P > 0.05$) in the microbial growth rate when compared to the notably used enrichment medium (Czapek Dox). However, the microbial growth rate on Czapek Dox significantly differed from that of other nitrogen sources ($P < 0.01$). More importantly, the final colony size or mycelial growth (84.9 ± 0.1 mm on day 10) on sodium glutamate significantly differs ($P < 0.001$) from that of other nitrogen sources.

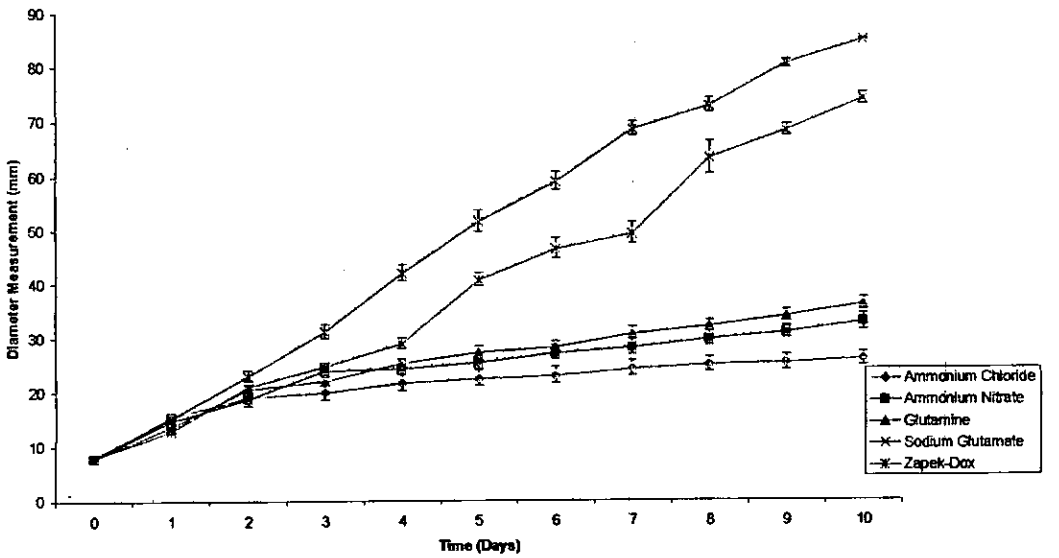

Figure 4.14: Growth Profile of *Curvularia pallescens* Boedijn on Different Types of Nitrogen Sources. Values are mean ± SEM for five replicate results.

The growth of *F. solani* in the nitrogen sources increased significantly over the growth period as compared to day zero ($P < 0.01$). The mycelial growth of *F. solani* was maximum, 85.00 mm with Czapek-Dox agar (ZA) on the 10^{th} day and minimum, 18.00 ± 0.73 mm with ammonium chloride (Figure 4.15).

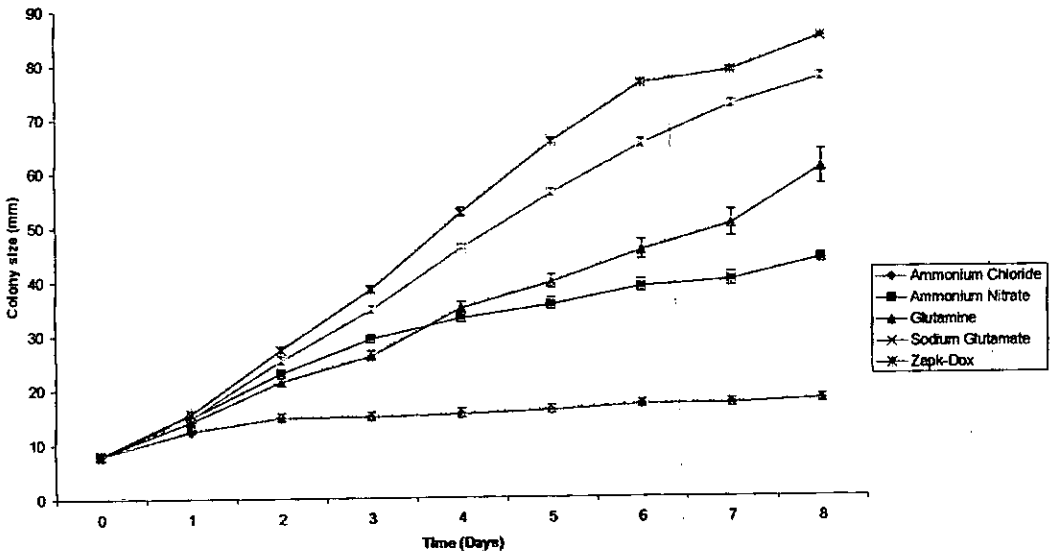

Figure 4.15: Growth Profile of *Fusarium solani* on Different Types of Nitrogen Sources. Values are mean ± SEM for five replicate results.

The fungus; *M. roridum* (IMI 394934) grew well in all the nitrogen sources (Ammonium nitrate, Glutamine, Sodium glutamate, Sodium nitrate and Urea) except in ammonium chloride (Figure 4.16). The growth on day 14 was highest; 68.20 ± 1.42 mm on sodium glutamate, 64.90 ± 0.94 mm on ammonium nitrate, 59.50 ± 0.27 mm and least; 10.20 ± 0.68 mm on ammonium chloride (Figure 4.16).

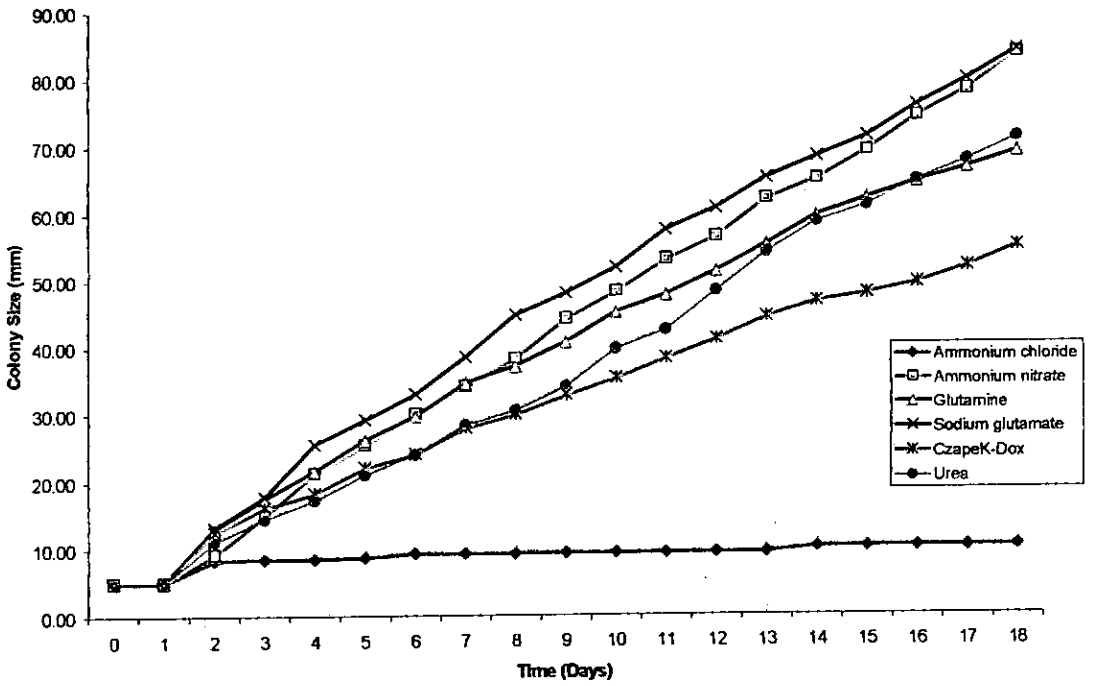

Figure 4.16: Growth Kinetics of *Myrothecium roridum* Tode (Culture IMI 394934) on Different Nitrogen Sources. Values are mean ± SEM for five replicate results.

There was no visible count in spores of *C. pallescens* on the different nitrogen sources except on ammonium chloride, which gave a concentration of $1.67E^{06} \pm 3.33E^{05}$ spores/ml (Figure 4.17). The spore count of *F. solani* was highest, $1.65E^{+08} \pm 2.9E^{+07}$ with sodium nitrate and minimum, $1.00^{+06} \pm 0.05E^{+06}$ with ammonium chloride (Figure 4.17). The spore concentration of *M. roridum* was maximum, 29.89×10^9 spores/ml on MEA and least, 0.003×10^8 spores/ml on ammonium chloride (Figure 4.17).

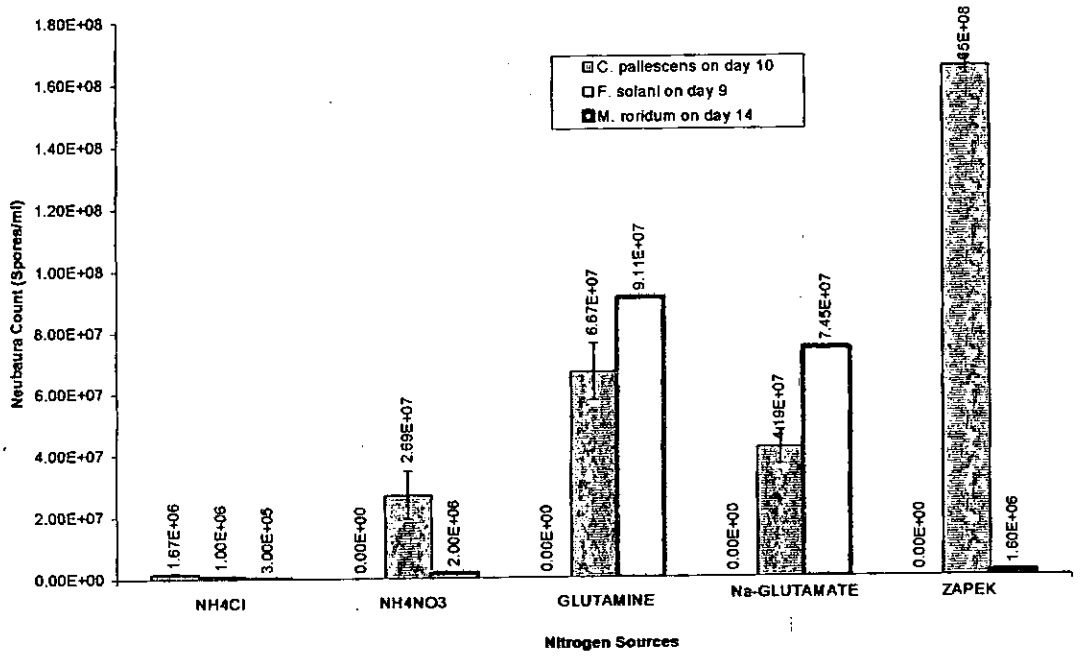

Figure 4.17: Neubaura count of Isolates on Different Types of Nitrogen Sources. Values are *mean* ± SEM *for five replicate results.*

4.10 Optimal pH for the Growth of the Organisms

In this study, the turbidometry analysis was used to determine the pH effect on microbial growth. Prior to the inoculation of the medium with the fungus, the initial pH of the autoclaved media (flasks labeled A to E) changed from 4.6 to 5.5, 5.8 to 6.6, 6.7 to 7.4, 7.8 to 8.2 and 9.0 to 8.6. The growth (Optical density) of *C. pallescens* was highest at pH 5.5 and least at pH 8.6 (Figure 4.18). The organism changed the pH of the medium within the two days of incubation. The pH was reduced from 5.5, 6.6, 7.4, 8.2 and 8.6 to 5.2, 5.9, 6.8, 7.7 and 7.8 respectively. The optimal growth pH of *F. solani* was 6.6 and the growth of this organism was least at pH 8.55 (Figure 4.18). And the pH of the growth medium was also reduced from 5.5, 6.6, 7.4, 8.2 and 8.6 to 5.5, 6.0, 6.3, 6.9 and 6.9 respectively. The optimal growth pH of *Myrothecium roridum* (IMI 394934) in this experiment was pH 5.5 (Figure 4.18). The pH of the growth medium was also reduced from 5.5, 6.6, 7.4, 8.2 and 8.6 to 5.3, 5.9, 7.1, 7.9 and 8.4 respectively.

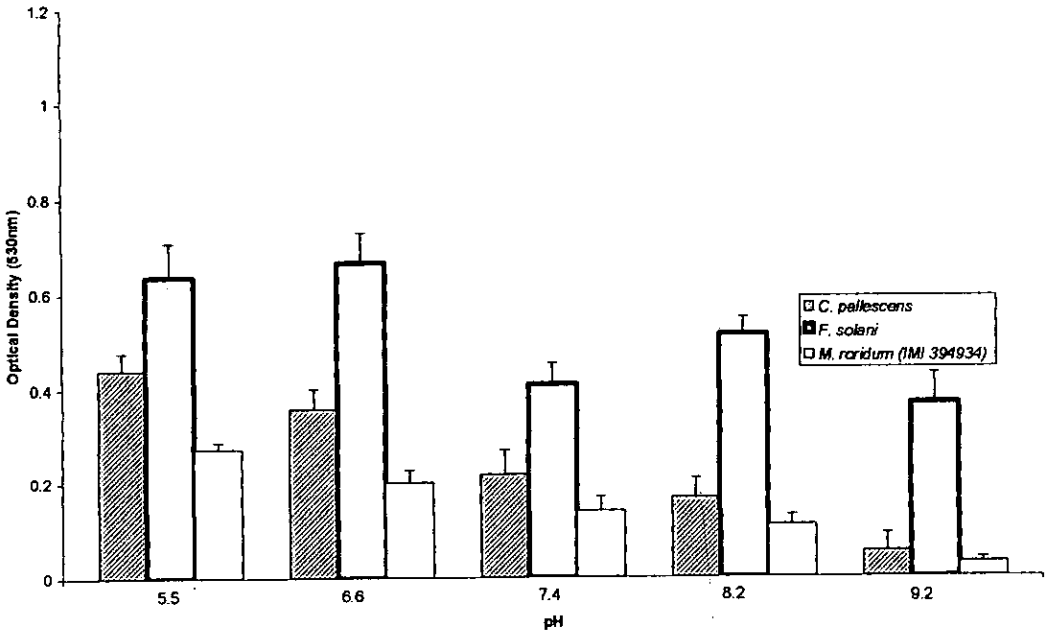

Figure 4.18: Optimal Growth pH for *C. pallescens*, *F. solani* and *Myrothecium roridum* Tode (IMI 394934). Values are mean ± SEM for triplicate results

4.11 Determination of the Cellulytic Activity of the Phytopathogens

The cellulolytic activity of the pathogens was determined using different carbon sources; carmoxymethylcellulose, sawdust and water hyacinth leaf. The result obtained (specific activity as µmole sugar released/mg protein/min.) showed that the fungi; *Curvularia pallescens* Boedjin, *Fusarium solani* and *Myrothecium roridum* (IMI 394934) produced cellulase and xylanase activity over the fermentation period. The exoglucanase and endoglucanase specific activity for *C. pallescens* was maximum, 18.96 ± 2.18 and 4.87 ± 0.14 on carboxymethylcellulose respectively; 12.08 ± 0.83 and 4.29 ± 0.22 on sawdust respectively, and least, 1.23 ± 0.02 and 0.53 ± 0.05 respectively on water hyacinth (Tables 4.3 – 4.5). The specific activity of β-glucosidase was highest, 7.23 on sawdust; 5.08 on carboxymethylcellulose and least, 0.36 on water hyacinth. Also, the xylanase specific activity (µmole sugar released/mg protein/min.) was highest, 11.90 ± 0.52 on carboxymethylcellulose, 5.42 ± 0.13 on sawdust and least, 1.34 ± 0.00 on water hyacinth (Tables 4.3 – 4.5). These data indicate that carboxymethylcellulose (CMC) was the best inducer of the cellulase complex in *C. pallescens* (Tables 4.3 – 4.5). The exoglucanase specific activity for *F. solani* was maximum, 15.10 ± 0.38 on carboxymethylcellulose, 6.56 ± 0.64 on sawdust and minimum, 1.22 ± 0.00 on water hyacinth. While the endoglucanase activity was highest, 4.84 ± 0.13 on carboxymethylcellulose, 1.77 ± 0.09 on sawdust and lowest, 0.43 ± 0.01 on water hyacinth (Tables 4.3 – 4.5). Also, the β-glucosidase specific activity was 3.15 on carboxymethylcellulose, 0.87 on sawdust and least, 0.50 on water hyacinth.

The xylanase specific activity was maximum, 8.00 ± 0.08 carboxymethylcellulose on day 16, 5.86 ± 0.20 on sawdust on day 12 and least; 1.30 ± 0.05 with water hyacinth on

day 8. These data also indicate that carxymethylcellulose (CMC) was the best inducer of the cellulase complex in *F. solani* (Tables 4.3 – 4.5).

The exoglucanase and endoglucanase specific activity for *Myrothecium roridum* (IMI 394934) was highest, 3.08 ± 0.12 and 2.75 ± 0.21 respectively on CMC, 1.93 ± 0.25 and 1.76 ± 0.09 on sawdust and least, 0.36 ± 0.06 and 0.41 ± 0.08 on WHB. Similarly, the β-glucosidase and xylanase specific activity were highest on CMC and least on WHB (Tables 4.3 – 4.5).

Table 4.3. Maximum Specific activity of Carboxymethylcellulose Induced Cellulases in Fungal Isolates

Isolates	Cellulase Specific Activity (μmol. sugar release/min./mg. protein)			
	Exoglucanase	Endoglucanase	β-glucosidase	Xylanase
Curvularia pallescens	18.96 ± 2.18	4.87 ± 0.14	5.08 ± 0.18	11.90 ± 0.52
Fusarium solani	15.10 ± 0.38	4.84 ± 0.13	3.15 ± 0.21	8.00 ± 0.08
Myrothecium roridum (IMI 394934)	3.08 ± 0.12	2.75 ± 0.21	11.2 ± 0.25	5.97 ± 0.04

The cultures were grown at 120 rpm and 25 ± 2°C for 16 days. Values are Mean ± SEM of Triplicate Results from independent experiment

Table 4.4. Maximum Specific activity of Sawdust Induced Cellulases in Fungal Isolates

Isolates	Cellulase Specific Activity (μmol. sugar release/min./mg. protein)			
	Exoglucanase	Endoglucanase	β-glucosidase	Xylanase
Curvularia pallescens	12.08 ± 0.83	4.29 ± 0.22	7.23 ± 0.19	5.42 ± 0.13
Fusarium solani	6.56 ± 0.64	1.77 ± 0.09	0.87 ± 0.90	5.86 ± 0.20
Myrothecium roridum (IMI 394934)	1.93 ± 0.25	1.76 ± 0.09	7.90 ± 0.28	4.69 ± 0.06

The cultures were grown at 120 rpm and 25 ± 2°C for 16 days. Values are Mean ± SEM of Triplicate Results from independent experiment

Table 4.5. Maximum Specific activity of Water hyacinth Induced Cellulases in Fungal Isolates

Isolates	Cellulase Specific Activity (μmol. sugar release/min./mg. protein)			
	Exoglucanase	Endoglucanase	β-glucosidase	Xylanase
Curvularia pallescens	1.23 ± 0.02	0.53 ± 0.05	0.36 ± 0.01	1.34 ± 0.00
Fusarium solani	1.22 ± 0.00	0.43 ± 0.01	0.50 ± 0.03	1.30 ± 0.05
Myrothecium roridum (IMI 394934)	0.36 ± 0.06	0.41 ± 0.08	4.4 ± 0.05	1.04 ± 0.04

The cultures were grown at 120 rpm and 25 ± 2°C for 16 days. Values are Mean ± SEM of Triplicate Results from independent experiment

4.12 Phytotoxin Production by the Three Isolates

The three organisms; *C. pallescens, F. solani* and *Myrothecium roridum* Tode (IMI 394934) were used for phytotoxins production on potato sucrose broth. The culture filtrate from *Myrothecium roridum* Tode (IMI 394934) produced a necrotic lesion at the site of infiltration on the water hyacinth leaves. This necrotic symptom which was similar to that produced by the fungus on the water hyacinth leaf extended both longitudinally and latitudinally over the incubation period as shown below (Figure 4.19). No disease symptom was observed in *C. pallescens* and *F. solani* culture fluid leaf bioassay. Hence, the effect of various parameters on the production of the phytotoxins by *Myrothecium roridum* (IMI 394934) was investigated.

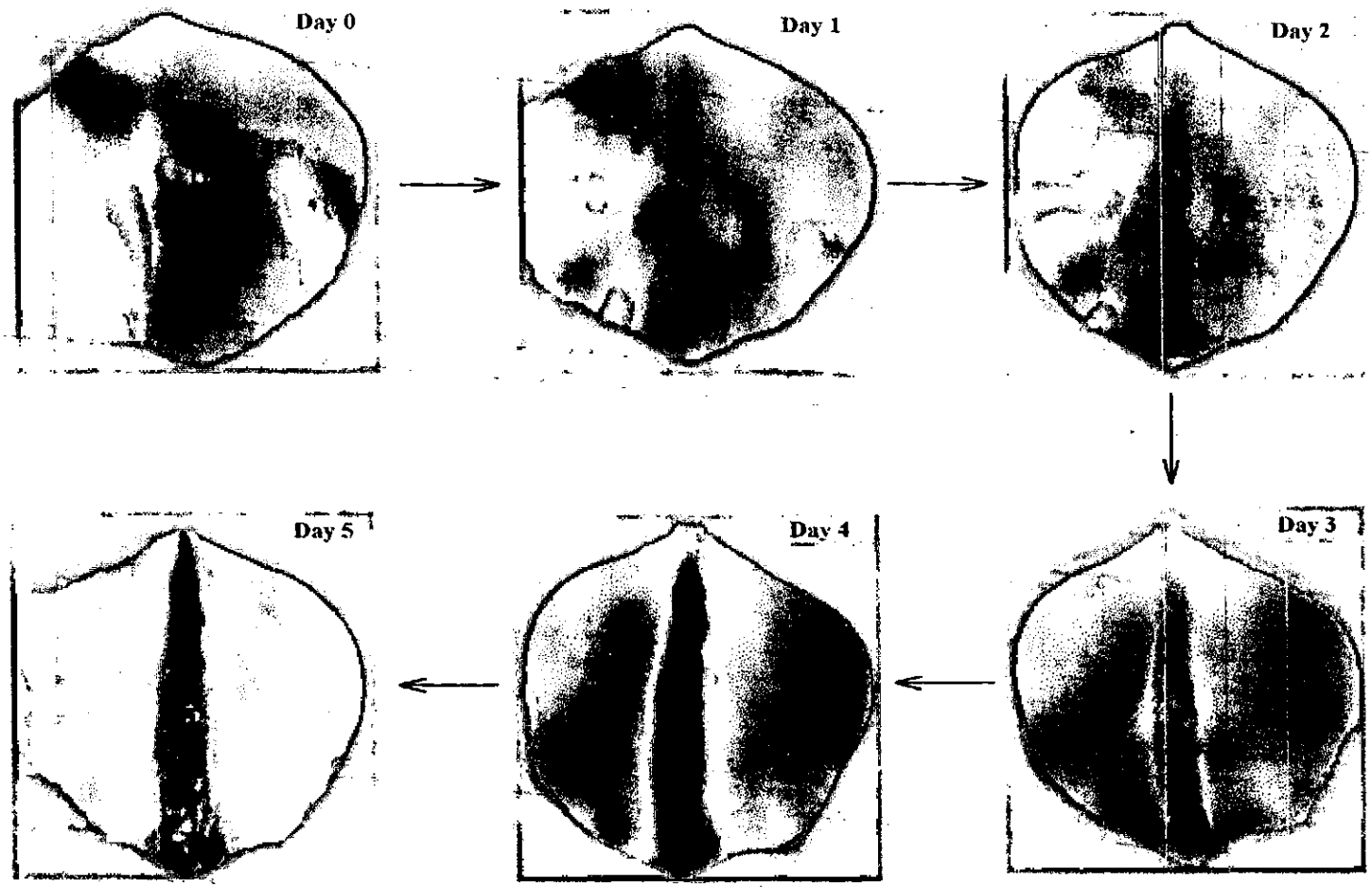

Figure 4.19: Lesions formed on punctured leaves of water hyacinth (*Eichhornia crassipes*) after infiltration of crude culture filtrate produced by *Myrothecium roridum* (IMI 394934), photographed over five days after infiltration.

4.13 Effect of Light Regimes on Phytotoxin Production

The effect of light on the phytotoxin production by *Myrothecium roridum* (IMI 394934) was examined under two different photoperiods; first under 24 hours dark periods for 25 days and the second for 25 days under 12 hours light and dark cycle. After the assay for biological activity of the culture filtrate produced by the organism, the result showed that the toxin was only produced in the 12 hours light and dark cycle. The average severity index (ASI) of the culture filtrate from the first experiment was 0.20 ± 0.00 while that of the second experiment was 4.00 ± 0.23 after 48 hours of culture infiltration on the detached leaves of water hyacinth.

4.14 Effect of pH on Toxin Production

The production of the phytotoxin by *M. roridum* (IMI 394934) over a pH range 4.0 to 7.5 was examined. The result obtained showed that the phytotoxin was produced over a pH range 4.5 to 7.5 but in varying degree. The average severity index (ASI) of the toxin produced at each pH range was increased after a dilution of the culture filtrate. The average severity index (ASI) of the toxin at 70% was maximum, 5.00 ± 0.00 at pH 4.5 3.00 ± 0.26 at pH 5.0, 3.50 ± 0.22 at pH 5.5, 4.5 ± 0.22 at pH 6.0, 3.50 ± 0.22 at pH 6.5, 4.50 ± 0.22 at pH 7.0 and 3.33 ± 0.33 at pH 7.5 (Table 4.6). The result showed that the ASI of the toxin produced at pH 4.5 was significantly greater than that obtained at pH 5.0, 5.5 and 6.5 ($P < 0.001$), this was also greater than that obtained at pH 7.5 ($P < 0.01$) and that obtained at pH 6.0 and 7.0 ($P < 0.05$) respectively.

More so, the final pH of the fermentation medium changed after toxin production at each pH range (Table 4.7). The final pH in each case changed to basic between a pH range of 8.1 and 8.7 except that at initial pH 4.0 (with no toxin produced) which changed to 5.2.

The biomass concentration (g/100ml) was least; 0.42 ± 0.00 at pH 4.0, 11.17 ± 0.71 at pH 4.5, 12.42 ± 0.94 at pH 5, 13.45 ± 1.02 at pH 5.5, 12.46 ± 0.91 at pH 6, 11.15 ± 0.89 at pH 6.5, 9.42 ± 0.93 at pH 7 and 10.14 ± 0.87 at pH 7.5 (Table 4.7). The result indicates that the maximum biomass production was achieved at pH 5.5. This result also showed that a negative correlation exists between the average severity index of phytotoxin and the pH of toxin production (Appendix). Although, this was not significant ($P > 0.05$). Similarly, a non-significant negative correlation existed between biological activity and biomass concentration ($P>0.05$).

Table 4.6: Response of detached leaves of water hyacinth (*Eichhornia crassipes*) to culture filtrate produced at different pH by *Myrothecium roridum* (IMI 394934) on potato sucrose broth, 48 h after inoculation (mean of six replicates).

pH	4.5	5	5.5	6	6.5	7	7.5
100%	2.33 ± 0.21	2.33 ± 0.33	2.00 ± 0.26	2.00 ± 0.26	2.00 ± 0.26	2.00 ± 0.37	0.50 ± 0.22
70%	**5.00 ± 0.00**	3.00 ± 0.26	3.50 ± 0.22	**4.50 ± 0.22**	3.50 ± 0.22	**4.50 ± 0.22**	3.33 ± 0.33
50%	**4.67 ± 0.21**	2.67 ± 0.21	3.00 ± 0.26	3.00 ± 0.26	3.00 ± 0.37	3.00 ± 0.00	3.00 ± 0.26
30%	3.00 ± 0.00	2.00 ± 0.26	3.00 ± 0.26	3.00 ± 0.37	2.00 ± 0.26	2.33 ± 0.21	2.67 ± 0.21
10%	2.00 ± 0.26	1.50 ± 0.22	2.50 ± 0.22	2.00 ± 0.26	2.67 ± 0.33	2.33 ± 0.33	1.67 ± 0.21
1%	1.67 ± 0.42	0.67 ± 0.21	2.50 ± 0.22	2.00 ± 0.26	0.00 ± 0.00	0.50 ± 0.22	1.50 ± 0.22

[1]Data represent Average Severity Index (ASI) ± S.E.M. derived from the following foliar symptoms scale: 0–5, where 0 = no symptoms, 1 = 1–10% of foliage with chlorosis and/or necrosis, 2 = 11–30%, 3 = 31–70%, 4 = 71–90%, and 5 = 91–100%.

Table 4.7: Initial and Final pH of Fermentation Medium and Biomass Concentration

Initial pH of Fermentation Medium	Final pH of Fermentation Medium	Biomass Concentration (g/L)
4	5.2	0.42 ± 0.00
4.5	8.1	11.17 ± 0.71
5	8.5	12.42 ± 0.94
5.5	8.7	13.45 ± 1.02
6	8.7	12.46 ± 0.91
6.5	8.3	11.15 ± 0.89
7	8.6	9.42 ± 0.93
7.5	8.4	10.14 ± 0.87

4.15 Effect of Media on Phytotoxin Production

The degree of phytotoxin production by *M. roridum* (IMI 394934) was investigated using seven different media types namely: malt extract broth (MAB), potato carrot broth (PCB), potato dextrose broth (PDB), potato sucrose broth (PSB), water hyacinth leaf broth (WHB), Sabouraud broth (SB) and zapek-Dox broth (ZDB). The result obtained showed that the phytotoxin was produced in varying amounts in the media types. The average severity index (phytotoxic activity or concentration) of the phytotoxin from each medium increased after the dilution of the crude toxin with distilled water (Table 4.8). The ASI of the various ranges of diluted toxin differed significantly ($P < 0.05$) from the undiluted filtrate in each medium except at 70% and 50% toxin concentration in PSB and 70% concentration in PDB. The highest biological activity was obtained at 70% toxin concentration with each medium except WHB (50% concentration). The average severity index (ASI) of the phytotoxin from each medium was 3.00 ± 0.37 with MAB, 3.33 ± 0.21 with PCB, 3.00 ± 0.26 with PDB, 1.67 ± 0.21 with PSB, 3.33 ± 0.33 with WHB, 2.67 ± 0.21 with SB and 2.33 ± 0.33 with ZDB. The maximum ASI/phytotoxin concentration was observed with PCB and WHB. This was not significantly different from that obtained from MAB and PDB ($P > 0.05$). However, it significantly differed from that obtained in SB and ZD ($P < 0.05$). Also, this was significantly different from that produced in PSB ($P < 0.001$).

Table 4.8. Response of detached leaves of water hyacinth (*Eichhornia crassipes*) to diluted culture filtrates from seven defined culture media, 48 h after inoculation (mean of six replicates).

Crude toxin Conc.	Non-autoclaved Media Types						
	MAB	PCB	PDB	PSB	WHB	SB	ZD
100%	0.00 ± 0.00	0.00 ± 0.00	0.67 ± 0.21	1.00 ± 0.26	1.67 ± 0.21	0.00 ± 0.00	0.00 ± 0.00
70%	**3.00 ± 0.37**	**3.33 ± 0.21**	**3.00 ± 0.26**	**1.67 ± 0.21**	3.00 ± 0.26	**2.67 ± 0.21**	**2.33 ± 0.33**
50%	2.67 ± 0.33	2.67 ± 0.21	2.33 ± 0.21	1.33 ± 0.21	**3.33 ± 0.33**	2.00 ± 0.26	1.67 ± 0.33
10%	2.67 ± 0.33	2.00 ± 0.26	0.33 ± 0.21	0.00 ± 0.00	2.6 ± 0.21	1.33 ± 0.21	0.33 ± 0.21
1%	2.33 ± 0.33	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	1.67 ± 0.21	1.00 ± 0.26	0.00 ± 0.00

[1]Data represent Average Severity Index (ASI) ± S.E.M. derived from the following foliar symptoms scale: 0–5, where 0 = no symptoms, 1 = 1–10% of foliage with chlorosis and/or necrosis, 2 = 11–30%, 3 = 31–70%, 4 = 71–90%, and 5 = 91–100%.

4.16 Effect of Carbon Source on Phytotoxin Production

The production of phytotoxin by *M. roridum* (IMI 394934) on Czapek-Dox broth containing seven different types of sugars (xylose, sucrose, fructose, maltose, glucose, lactose and galactose) as carbon source was evaluated. The result obtained revealed that all the sugar types were capable of inducing toxin production but with varying capacity. The ASI (measured as diameter of necrotic zone on leaves) was 35.00 ± 4.85 with xylose, 21.50 ± 2.54 with sucrose, 14.88 ± 0.58 with fructose, 10.00 ± 0.89 with maltose, 19.38 ± 1.99 with glucose, 31.67 ± 1.34 with lactose and 25.17 ± 2.75 with galactose (Figure 4.20). The maximum ASI as obtained with xylose sugar was not significantly greater than that obtained with lactose or galactose ($P > 0.05$) but significantly greater than that obtained when sucrose or glucose was used ($P < 0.05$). Also this was significantly greater than that obtained from fructose ($P < 0.01$) and maltose ($P < 0.001$). More so, the ASI obtained from the second best phytotoxin inducing sugar; lactose was not significantly different from galactose ($P > 0.05$) but significantly different from that obtained when sucrose, fructose, maltose or glucose was used as carbon source ($P < 0.001$).

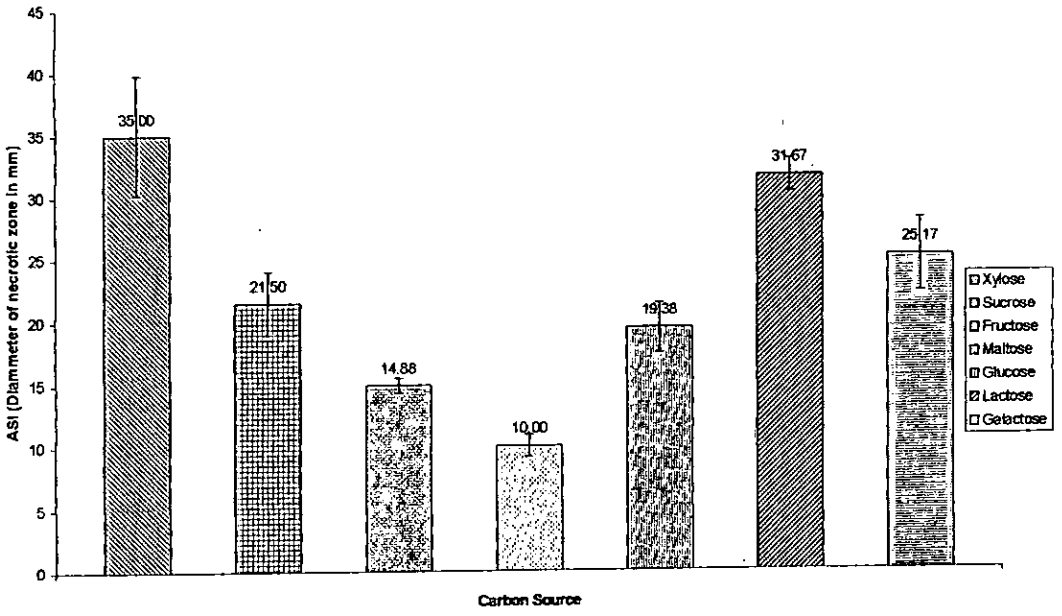

Figure 4.20: Dependence of Phytotoxin Production by *Myrothecium roridum* (IMI 394934) on Carbon Source. Values are mean ± SEM for six replicate results.

4.17 Effect of Exogenous Nitrogen Source on Phytotoxin Production

The influence of exogenous nitrogen source on *M. roridum* in phytotoxin production was investigated using Czapek-Dox broth containing different nitrogenous compounds such as yeast extract, ammonium nitrate, ammonium chloride, sodium glutamate, glutamine, urea and sodium nitrate as the sole nitrogen source. The results obtained showed that all these compounds were able to support phytotoxin production but in varying degrees. The diameter of the necrotic zone on the water hyacinth leaf which was the measure of inoculated toxin concentration was maximum; 30.50 ± 5.03 for glutamine, 28.88 ± 3.95 for sodium glutamate, 25.25 ± 1.11 with urea, 24.38 ± 0.76 for ammonium nitrate, 19.88 ± 3.43 for ammonium chloride and least; 13.75 ± 2.37 for yeast extract (Figure 4.21). The maximum phytotoxin concentration as obtained with glutamine was not significantly different from that obtained with sodium glutamate, urea, ammonium nitrate or ammonium chloride ($P > 0.05$) but was significantly greater than that obtained with sodium nitrate or yeast extract. More so, the phytotoxin concentration obtained from the second best nitrogen compound; sodium glutamate as compared to others was significantly greater than that obtained with sodium nitrate or yeast extract ($P < 0.05$).

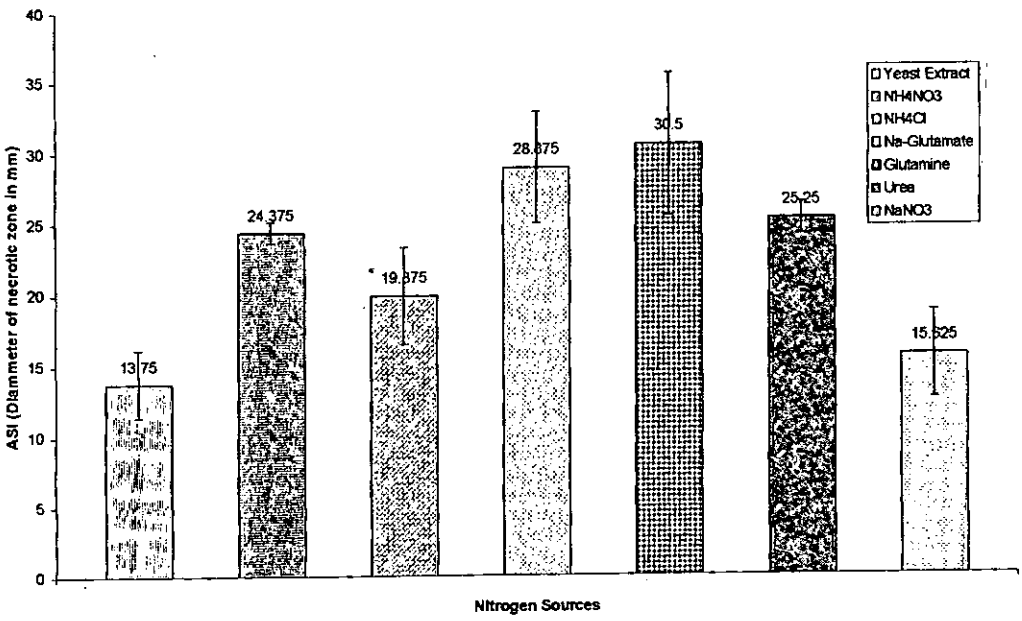

Figure 4.21: Depence of Phytotoxin Production *Myrothecium roridum* (IMI 394934) on Nitrogen Source. Values are mean ± SEM for six replicate results.

4.18 Effect of Light Regimes on the Potency of Phytotoxin Activity

The effect of photoperiod on biological activity of crude toxin produced by *M. roridum* (IMI 394934) on water hyacinth leaves bioassay under both condition of 24 hours darkness and 12 hours light and dark cycle was investigated. The crude toxin was adjusted over a concentration range 0.01 to 2.0% (w/v) before use. The ASI of the leaves in the dark was highest, 5.00 ± 0.00 at 2% toxin concentration and least; 2.0 at 0.02% and 0.01% concentration respectively (Figure 4.22). This maximum ASI at 2% concentration under dark reaction was significantly greater than those obtained at reduced concentrations ($P < 0.001$). While the ASI of the leaves under 12 hours light and dark regimen was maximum 5.00 ± 0.00 at 0.5% crude toxin concentration and least 1.67 ± 0.21 at 0.01% toxin concentration (Figure 4.22). This maximum ASI obtained at 0.5% crude toxin concentration was significantly greater than those obtained at 2%, 1%, 0.25%, 0.02%% and 0.01%, respectively ($P < 0.001$). The results indicate that the biological activity of the phytotoxin was more effective in the light over a concentration range of 0.02 to 1.00% as compared to that obtained in the dark experiment. The ASI of the leaves in light reaction was significantly greater than that obtained in the dark reaction at a concentration range 0.5%, 0.25% or 0.02% ($P < 0.05$). on the other hand, the ASI of the leaves in the dark reaction was significantly greater than that in the light reaction at 2% concentration ($P < 0.001$).

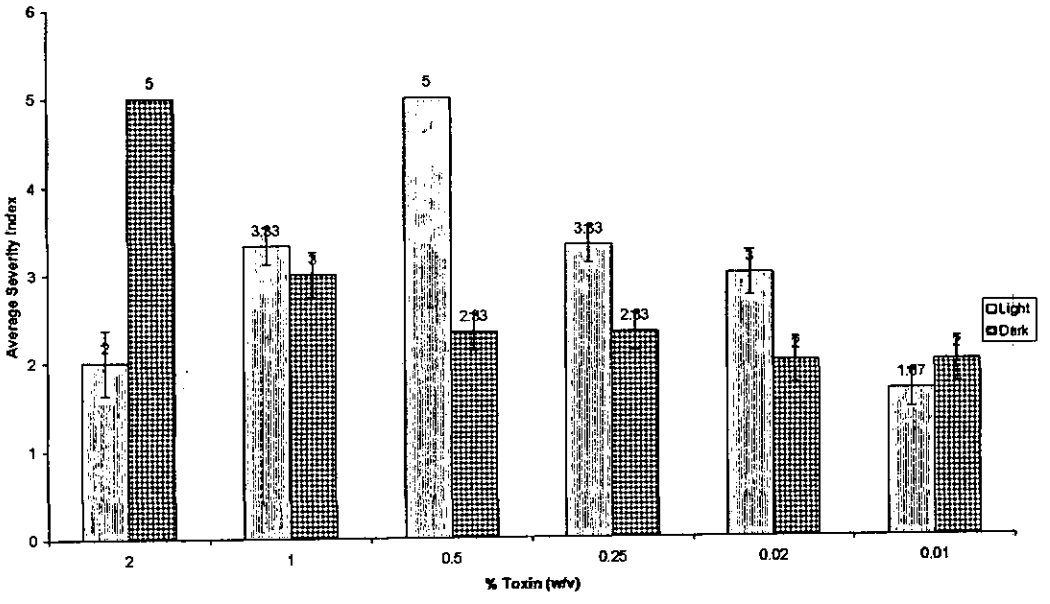

Figure 4.22: Effect of Photoperiod on Biological Activity of Crude Toxin. Values are mean ± SEM for six replicate results.

4.19 Effect of pH on the Potency of Phytotoxin Activity

The phytotoxin produced by *M. roridum* (IMI 394934) was adjusted to varying pH between 4.0 and 7.5 and the biological activity was determined in a water hyacinth leaves bioassay. The results obtained showed that the biological activity was exhibited between the pH ranges 4.5 to 7.5 at varying degree of ASI. The maximum ASI was 3.67 ± 0.33 at pH 6.0, 2.33 ± 0.21 at pH 7.5, 1.67 ± 0.21 at pH 5.0, 1.67 ± 0.33 at pH 6.5 and least; 0.83 ± 0.17 at pH 7.0 (Figure 4.23). The maximum ASI observed at pH 6.0 was significantly greater than ASI at the other respective pH's ($P < 0.01$).

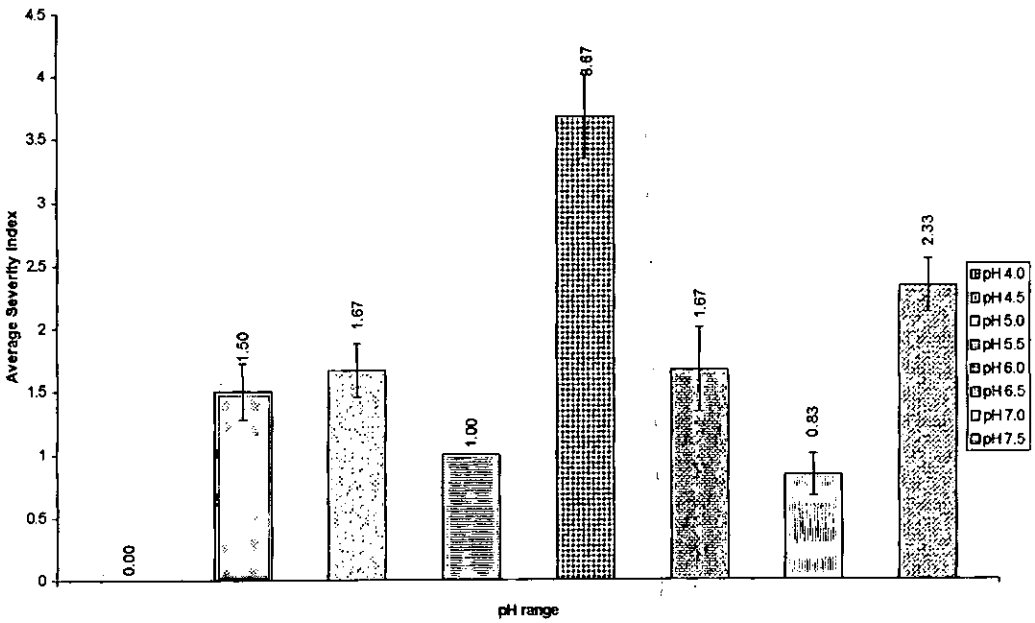

Figure 4.23: pH Dependence of *M. roridum* (IMI 394934) Phytotoxin on Biological Activity. Values are mean ± SEM for five replicate results.

4.20 Effect of Heat on the Potency of Phytotoxin Activity

The effect of heat on phytotoxin produced by *M. roridum* (IMI 394934) was investigated by comparing the biological activity of the non-autoclaved crude toxin with the autoclaved (121°C for 20 min.) toxin in a water hyacinth leaf bioassay. The results obtained showed that ASI of the autoclaved phytotoxin were mostly higher than the non autoclaved in all the media types used (MAB, PCB, PDB, PSB, WHB, SB and ZDB) (Table 4.9). The biological activity of the autoclaved culture filtrate in each medium was observed to increase after the dilution with distilled water. The maximum activity was observed at 70% crude toxin concentration in the media types except in WHB which had a maximum biological activity at 50% toxin concentration. The maximum ASI, 4.67 ± 0.21 observed with the autoclaved PCB or PDB at 70% concentration was significantly greater than that observed in the autoclaved PSB ($P < 0.001$) or ZDB ($P < 0.01$). These results indicate that the phytotoxin produced by *M. roridum* (IMI 394934) was heat stable and the activity was increased after autoclaving and diluting the culture filtrate.

Table 4.9: Effect of heat on different culture filtrates produced by *Myrothecium roridum* (IMI 394934) on detached water hyacinth leaves, 48 h after inoculation (mean of six replicates).

Crude toxin Conc.	Autoclaved Media Types						
	MAB	PCB	PDB	PSB	WHB	SB	ZD
100%	2.33 ± 0.33	1.33 ± 0.21	1.00 ± 0.26	1.33 ± 0.21	1.67 ± 0.33	1.00 ± 0.26	0.33 ± 0.21
70%	**4.33 ± 0.21**	**4.67 ± 0.21**	**4.67 ± 0.21**	**2.33 ± 0.21**	3.33 ± 0.33	**4.00 ± 0.26**	**3.33 ± 0.33**
50%	3.67 ± 0.33	3.67 ± 0.33	3.67 ± 0.33	2.33 ± 0.21	**4.00 ± 0.37**	3.67 ± 0.33	1.67 ± 0.21
10%	1.33 ± 0.21	3.00 ± 0.26	0.33 ± 0.21	2.33 ± 0.33	2.67 ± 0.21	0.00 ± 0.00	1.33 ± 0.21
1%	0.67 ± 0.21	2.00 ± 0.26	0.00 ± 0.00	0.00 ± 0.00	2.33 ± 0.42	0.00 ± 0.00	1.00 ± 0.26

[1]Data represent Average Severity Index (ASI) ± S.E.M. derived from the following foliar symptoms scale: 0–5, where 0 = no symptoms, 1 = 1–10% of foliage with chlorosis and/or necrosis, 2 = 11–30%, 3 = 31–70%, 4 = 71–90%, and 5 = 91–100%.

4.21 Biological Activity of Phytotoxin on Leaves of Various Plants

The toxicity of culture extract (concentration: 50%) of *M. roridum* (IMI 394934) was examined on various plant species. Four of the twenty six species of plants tested were susceptible to the toxin at varying degrees. The results are as presented in the Table 4.10 and figure 4.24 – 4.27 below.

Table 4.10a. Phytotoxin activity of culture fluid of *M. roridum* (IMI 394934) on various plant species 3days post toxin infilteration.

SN	Plants (Common Names)	Botanical Names	ASI	Comment
1.	Acalypha	*Acalypha cordifolia*	0.00 ± 0.00	
2.	Aloe vera	*Aloe vera*	0.00 ± 0.00	
3.	Amarantus	*Amaranthus viridis*	0.00 ± 0.00	
4.	Banana	*Musa sapientum*	0.00 ± 0.00	
5.	Beans	*Vigna unguiculata*	2.33 ± 0.33	Necrotic
6.	Cabbage	*Brassica oleracea*	0.00 ± 0.00	
7.	Carrot	*Daucus carota*	0.00 ± 0.00	
8.	Cassava	*Manihot esculentum*	0.00 ± 0.00	
9.	Cocoyam	*Colocasia esculentum*	0.00 ± 0.00	
10.	Corn	*Zea mays*	0.00 ± 0.00	
11.	Date palm	*Phoenix dactylifera*	0.00 ± 0.00	
12.	Long-fruited Jute	*Corchorus olitorius*	2.00 ± 0.58	Necrotic
13.	Garden egg	*Solanum melongena*	0.00 ± 0.00	
14.	Groundnut	*Arachis hypogea*	2.67 ± 0.33	Necrotic
15.	Lemon grass	*Cymbopogun citratus*	2.33 ± 0.33	Necrotic
16.	Lettuce	*Lactuca taraxacifolia*	0.00 ± 0.00	
17.	Mango	*Mangifera indica*	0.00 ± 0.00	
18.	Okro	*Hibiscus esculentus*	0.00 ± 0.00	
19.	Pawpaw	*Carica papaya*	0.00 ± 0.00	
20.	Pineapple	*Ananas comosus*	0.00 ± 0.00	
21.	Red savina	*Capsicum chinense*	0.00 ± 0.00	
22.	Plumed celosia	*Celosia argentea*	0.00 ± 0.00	
23.	Chili pepper	*Capsicum annuum*	0.00 ± 0.00	
24.	Tomato	*Lycopersicum esculentus*	0.00 ± 0.00	
25.	Water melon	*Citrullus lanatus*	0.00 ± 0.00	
26.	Yam	*Dioscorea alata*	0.00 ± 0.00	

[1]Data represent Average Severity Index (ASI) ± S.E.M. derived from the following foliar symptoms scale: 0–5, where 0 = no symptoms, 1 = 1–10% of foliage with chlorosis and/or necrosis, 2 = 11–30%, 3 = 31–70%, 4 = 71–90%, and 5 = 91–100%.

Table 4.10b. Phytotoxin activity of culture fluid of M. roridum (IMI 394934) on water hyacinth leaf 3days post toxin infilteration.

SN	Plant	Botanical Names	ASI	Comment
1	Water hyacinth	*Eichhornia crassipes*	5.00 ± 0.15*	Necrotic

[1]Data represent Average Severity Index (ASI) ± S.E.M. derived from the following foliar symptoms scale: 0–5, where 0 = no symptoms, 1 = 1–10% of foliage with chlorosis and/or necrosis, 2 = 11–30%, 3 = 31–70%, 4 = 71–90%, and 5 = 91–100%.

*maximum ASI achievable.

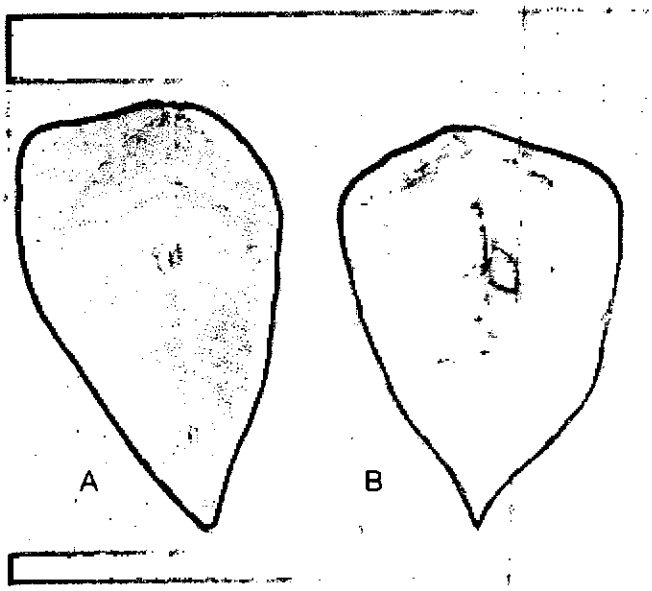

Figure 4.24: Phytotoxin activity of culture fluid of *M. roridum* (IMI 394934) on Beans. A= Control, B=Test

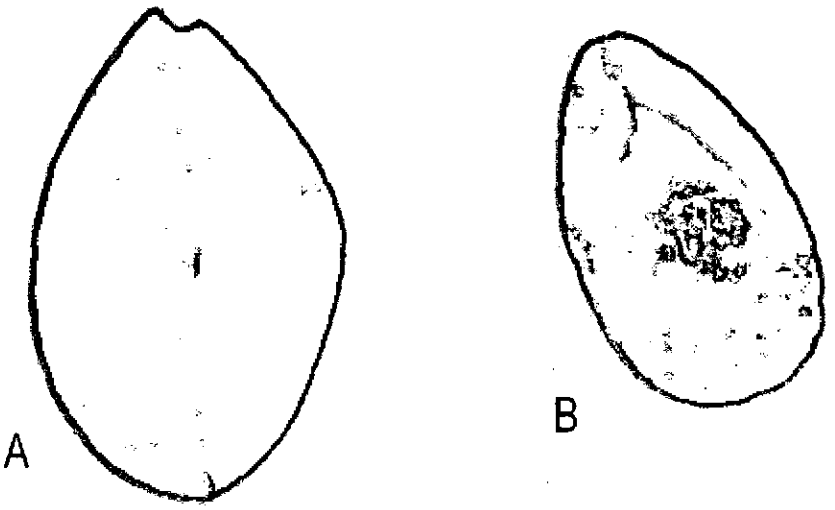

Figure 4.25: Phytotoxin activity of culture fluid of *M. roridum* (IMI 394934) on Groundnut. A= Control, B=Test

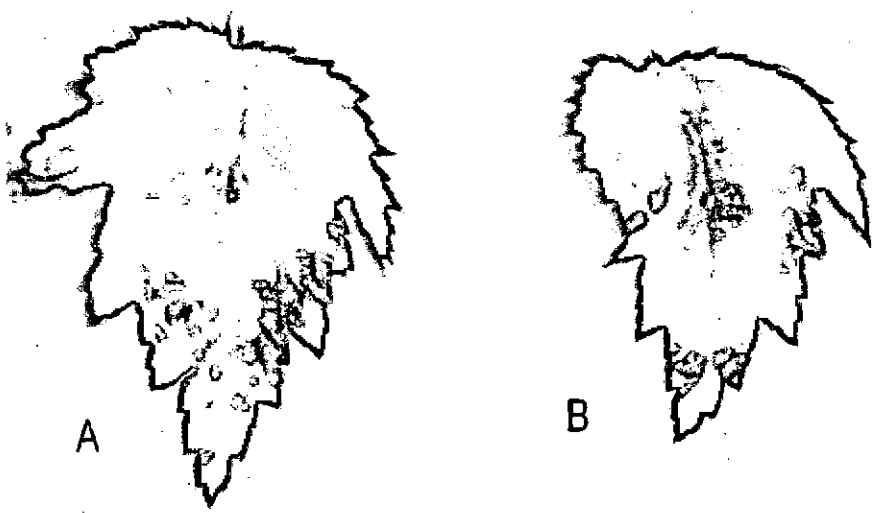

Figure 4.26: Phytotoxin activity of culture fluid of *M. roridum* (IMI 394934) on Long-fruited Jute. A= Control, B=Test

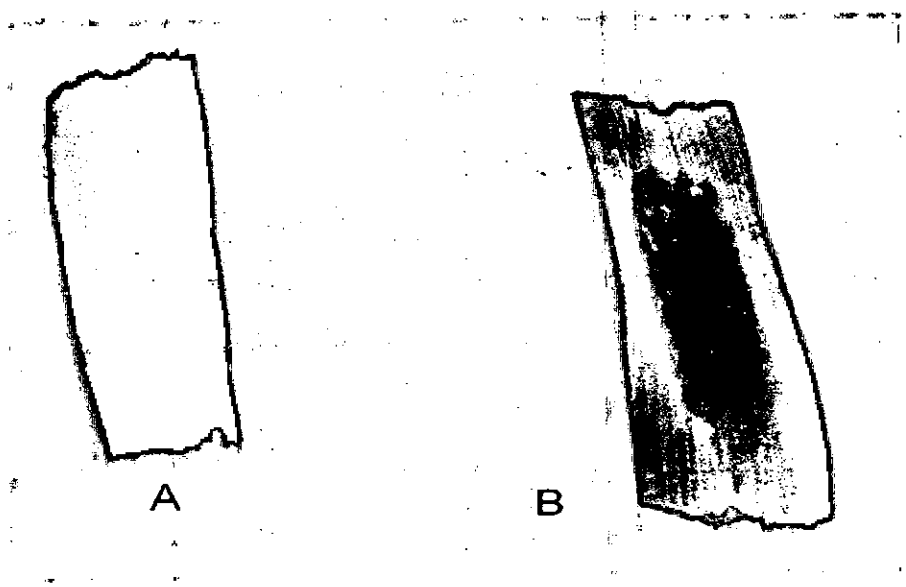

Figure 4.27: Phytotoxin activity of culture fluid of *M. roridum* (IMI 394934) on Lemon grass. A= Control, B=Test

4.22 Separation, Isolation and Partial Characterization of Toxins

Selection of developing solvent for the resolution of crude toxin metabolites was done prior to the extraction of the crude toxin. The lyophilized sample of the crude toxin was dissolved in methanol and resolved on a TLC plate using ethylacetate: methanol (9:1) and ethylacetate: methanol: water: acetic acid (10:1.7:1.3:0.2) as the mobile phase. The results obtained showed that there were four different fractions in each experimental set-up as against the control (uninoculated medium) (Figure 4.28). The fractions appeared to be more resolved in ethylacetate: methanol: water: acetic acid (EMWA) as compared to ethylacetate: methanol (EM) (Figure 4.28). The R_f values of the various fractions are as presented in the Table 4.11 below. Subsequently, the crude toxin was extracted in chloroform: methanol (1:1), evaporated in a rotary evaporator and separated under vacuum chromatography with dichloromethane, methanol: dichloromethane (10:90) and methanol: dichloromethane (20:80) used independently for the elution. The three eluents (1 to 3) obtained were spotted on a precoated analytical grade TLC plate and developed using: ethylacetate: methanol: water: acetic acid (10:1.7:1.3:0.2), methanol: dichloromethane (8:92) and methanol: dichloromethane: hexane (4:92:4). The results obtained showed that these solvents are capable of separating the metabolites at varying degrees and to a different number of fractions (Figure 4.29). The results also indicate that methanol: dichloromethane (8:92) was the best solvent to resolve the eluent 1 and 2 as more fractions with clear separations were observed (Appendix; Table 4.12 - 4.14). Although, eluent 3 gave 6 fractions as compared to 7 fractions each on ethylacetate: methanol: water: acetic acid and methanol: dichloromethane: hexane respectively. Fractions on the TLC plate resolved in methanol: dichloromethane were eluted in

distilled water and used for biological assay on water hyacinth leaf. The results showed that the fractions with R_f value 0.51 in eluent 1, 2 and 3 (Appendix; Table 4.13) only caused necrotic symptom on the leaves. This symptom was similar to that caused by the fungus and also the crude toxin on the water hyacinth leaves. The purity check on this fraction was confirmed when it was eluted and spotted on the TLC plate as it gave a single band with R_f value 0.51.

Weight of the lyophilized phytotoxic fraction = 1.2 mg

Total weight of sample applied to TLC plate = 293.1 mg. Yield = 0.4094%

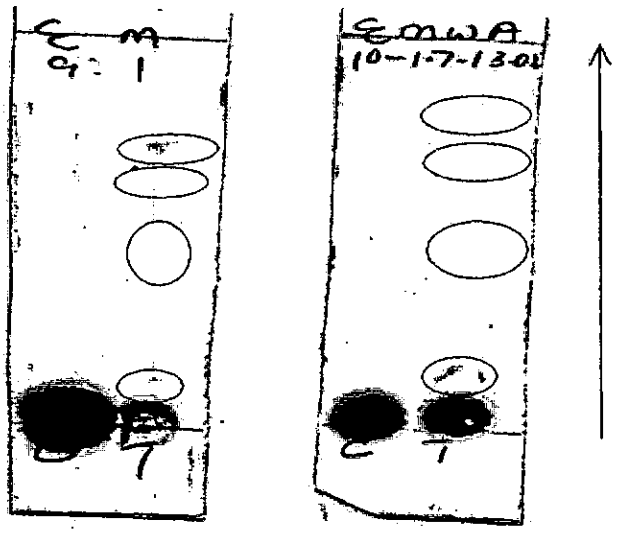

Figure 4.28: Thin Layer Chromatogram of Toxin Metabolites produced by *Myrothecium roridum* (IMI 394934) in Ethylacetate: methanol (9:1) and Ethylacetate: methanol: water: acetic acid (10:1.7:1.3:0.2) after Vanillin Sulphuric acid reaction

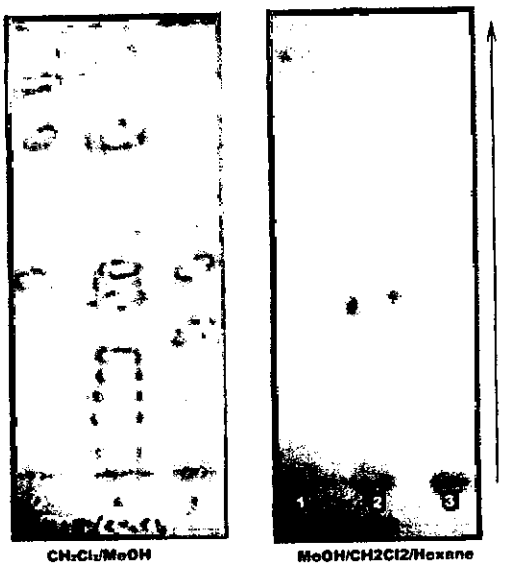

Figure 4.29: Thin Layer Chromatogram of Toxin Metabolites produced by *Myrothecium roridum* (IMI 394934) in Methanol: dichloromethane (8:92) and Methanol: dichloromethane: Hexane (4:92:4) before Vanillin Sulphuric acid reaction.

CHAPTER FIVE

5.0 DISCUSSION

Survey for Phytopathogens

Ten sites each in four different locations in Lagos and its environs were sampled for infested water hyacinth plant. A unique pattern of disease symptom was observed with water hyacinth infested with *Myrothecium roridum* (IMI 394934). Symptom or diseased water hyacinth plants were peculiar to two sample locations; Badagry Creeks (N6.41950° E2.86019°, N6.42066° E2.86630° and N6.41748° E2.87552°) and Ogun River (Isheri: N6.64091° E3.8406°). The diseased plants were observed on a seasonal basis for the three years of survey in Badagry when the creeks were densely covered with water hyacinth. Similarly, infested plants were observed in the last two years of study in Ogun river (Isheri), although, the infested plants in Ogun river were scanty. The Lagos Lagoon is a salty water body and is usually poorly infested with water hyacinth until the rainy season returns when salinity drops to minimal. Previous explorative studies in Nigeria have shown the presence of some fungal isolates such as *Cercospora rodmanii* Conway, *Cladosporium oxysporum* Berk. & Curt and *Phyllosticta* sp. on water hyacinth (Barreto and Evans, 1996). In this study, a different fungal species identified as *Myrothecium roridum* (IMI 394934) on water hyacinth in Nigeria was obtained. Reports indicate that this organism has been isolated in India, Malaysia (IMI 277583), Mexico and Philippines Thailand/Burma (IMI 79771) (IMPECCA Technical Guide Series No. 1, 2001; Charudattan, 2001; Barreto and Evans, 1996). However, we have not come across such documented report of this isolate on water hyacinth in Africa.

Fungal Isolation from Diseased Water Hyacinth Tissue

The cursory observation of the fungal isolation was that improper sterilization with about 0.26% sodium hypochlorite made it difficult for the isolation of the pathogenic fungus. At this concentration of sterilant, the non-pathogenic fungal species grew exponentially and overshadowed the pathogen of interest. However, at a higher concentration of the sterilizing solution, the contaminants were reduced and the pathogen of interest was observed to appear first in the culture plate. Two different isolates; *Fusarium* and *Myrothecium* species appeared whitish and fluffy morphologically on potato dextrose agar plate and were difficult to distinguish at the early stage of the isolation and with *Fusarium* growing at a much faster rate and overshadowing the *Myrothecium*. The use of a 1.4% sterilant resulted in the inhibition of the growth of *Fusarium* and the emergent of *Myrothecium*. Literature have reported the use of more than 1% sodium hypochlorite solution as a sterilant in plant tissue culture experiments (Chanpramel *et al.*, 1996; Yildiz and Er, 2002; Zheng *et al.*, 2002; Coombs and Franco, 2003; Flores *et al.*, 2006) as against 0.26% concentration used in the early part of isolation in this study. This suggests that a proper sterilization of plant tissue prior to tissue culture is very important in eliminating contaminants in plant materials.

Pathogenicity Screening

As a result of pathogenicity testing and on the basis of disease severity, *M. roridum* (IMI 392934) was found to be highly destructive on water hyacinth. This fungus has been earlier implicated as a potential biocontrol agent of the water hyacinth plant in India, Philippines, Malaysia and Mexico (Barreto and Evans, 1996; IMPECCA Technical Guide No 1, 2001). Of the nearly 70 fungi and bacteria recorded on water hyacinth (Barreto and

Evans, 1996; Charudattan, 1996), only about 15 have been adequately tested and confirmed to be highly virulent pathogens. Of these, three fungal pathogens, *Acremonium zonatum, Alternaria eichhornia*, and *Cercospora piaropi* (= *C. rodmanii*), have been studied intensively as biocontrol agents and shown to be effective in controlling water hyacinth under experimental conditions (Martyn and Freeman, 1978; Charudattan *et al.*, 1985; Shabana *et al.*, 1995b; Charudattan, 2001). This leaves a large number of the other reported fungi and bacteria to be assessed for their biocontrol potential (Charudattan, 2001). Thus, for now, the choice of pathogens for biological control is limited to Uredo eichhorniae, as a classical biocontrol agent, and as bioherbicide agents namely; *A. zonatum, A. eichhorniae, C. piaropi* (= *C. rodmanii*), *Myrothecium roridum*, and *Rhizoctonia solani* (Charudattan, 2001). Also, Bateman (2001) reported that scientific collaborators of International monitoring programme on *Eichhornia crassipes* (IMPECCA) have identified a short list of fungal species that show most promise in their potential as mycoherbicides for water hyacinth control in Africa. In order of preference based on the virulence, they are: 1) *Alternaria eichhorniae;* 2) *Acremonium zonatum*; 3) *Cercospora piaropi/C. rodmanii;* 4) *Rhizoctonia solani* and *Alternaria alternate*; 5) *Myrothecium roridum*. In this study, *M. roridum* (IMI 394934) showed a much greater disease incidence and disease severity compared to that recently reported (El-Morsy *et al.*, 2006) on *Alternaria alternata*. Healthy water hyacinth in our study appeared with dead lamina and submerged petiole 4weeks post-inoculation with *M. roridum* (IMI 394934), while a formulation of spores of *A. alternata* strain in a previous report gave values of disease incidence, 80% and disease severity, 95% following 60 days of infection (El-Morsy *et al.*, 2006). *Curvularia pallescens* Boedjin and *Fusarium solani*

isolated in this study were not virulent and not considered potential candidate for water hyacinth control. Although, these two isolates have also been reported to be found associated with water hyacinth in some countries but were reported as non virulent to be considered for mycoherbicide development for water hyacinth (Barreto and Evans, 1996; El-Morsy *et al.*, 2006).

Histopathology of Diseased Water Hyacinth Plant

The most pronounced anatomical feature of this plant was the presence of gas filled chambers. Air chambers are large, usually regular (circular to hexagonal) intercellular spaces extending through leaf and long distances through stem. The reduction in the number of air chamber could lead to a reduction in respiratory and photosynthetic process in the leaves. These chambers provide a sort of internal atmosphere for the plant. (Mahmood *et al.*, 2005). The cross partition of air chambers are called diaphragms, perhaps they function to prevent flooding. Air chambers also give buoyancy to the organs in which they occur (Mahmood *et al.*, 2005).

Xylem lacuna and the phloem in the vascular system were reduced in the infected leaves. This implies that the transport of minerals and water by the xylem and also the transport of food by the phloem are hampered. Therefore, cell growth ceases and there is no replacement of dead or worn out cells.

Host specificity of *M. roridum* (IMI 394934) on Some Crops

The fungus, *M. roridum,* has been reported on some host plants which include; *Antirrhinum, Coffea, Cucurbitaceae, Cyamopsis psoraloides, Gardenia, Gloxinia, Gossypium, Hibiscus esculentus, Hypocyrta, Lycopersicon esculentum, Molucella, Sesamum indicum, Solanum melongena, Trifolium pretense, Vigna unguiculata, Vinca,*

Viola (IMPECCA Technical Guide No. 1, 2001). Recently, scientists gave the first report of *Myrothecium roridum* causing *Myrothecium* Leaf Spot on *Salvia* spp. in the United States (Mangandi *et al.*, 2007). Also, Srivastava and Khan (1997) reported that soybean crop inoculated with *Myrothecium roridum* on different days after sowing exhibited varying degrees of disease severity (44.4 to 83.3%) and yield losses (7.6 to 20.4%). The fungus *Myrothecium roridum* was first reported to be pathogenic to muskmelons (*Cucumis melo*) in 1961 by McLean and Sleeth (Mackay *et al.*, 1994). Assays have been developed for assessing the safety of non-target host plants against pathogens (Morris *et al.*, 1999; Charudattan, 1996; Mohan Babu *et al.*, 2002) and were used in this study against 26 plant species. In this study however, none of the 26 test plants showed any disease symptom to the strain of *Myrothecium roridum* (IMI 394934) employed. Other fungal agents which have worldwide use as biocontrol agents of water hycinth have also been reported to infect a varied number of economical and ornamental plants (IMPECCA Technical Guide No. 1, 2001; Nag Raj and Ponnappa 1970).

Effect of Media Types on Growth

Sonia *et al.*, (1998) and Tabassam *et al.*, (2003) have reported potato dextrose agar as the best medium for the growth of *Curvularia pellescens* Boedijn. In this study however, our isolate of *Curvularia pellescens* Boedijn grew best on WHA when compared to that of the other media types which include potato dextrose agar. This may be due to the nutritional combination present in water hyacinth leaf, which may have also made the plant the natural host for the organism. Proximate analyses of the water hyacinth leaves by Igbinosun *et al.* (1988) showed that it contains 14.7% dry matter, 12.4% ash, 22.75% crude protein, 15.0% crude fibre and 4.82% lipid. Igbinosun *et al.* (1988) also reported

the mineral composition of the leaf as 0.44% phosphorus, 4.28% potassium, 0.02% sodium, 2.63% calcium, 190.5 ppm magnesium, 77.3 ppm manganese, 77.3 ppm zinc and 1.62 ppm.

The mycelial growth was highest on WHA and least on TWA while the spore concentration was highest on TWA and lowest on WHA. This result infers that the sporulation of *C. pallescens* on WHA and TWA is inversely proportional to their mycelial growth. It also suggests that a less nutritive medium is needed for the sporulation of *C. pallescens*. Also, WHA gave the best mycelial growth support to *F. solani,* while the sporulation was best on PDA and least on SA. This also indicates that a more nutritive and a less nutritive medium is needed for mycelial growth and sporulation of these fungi respectively.

However, the mycelial growth of *Myrothecium roridum* (IMI 394934) was highest on TWA (the least nutritive medium) and least on ZA. This is contrary to the mycelial growth performance of *C. pallescence* and *F. solani* on TWA and WHA. Also the growth rate of the organisms showed the broad mycelial growth performance on a comparative basis on media types. *M. roridum* (IMI 394934) which was the only pathogenic isolate had the highest growth rate on TWA when compared to *C. pallescence* and *F. solani.* This suggests that in order to keep the fast growing non pathogenic organisms in check TWA could be used for the isolation of a monoculture of *M. roridum* from the diseased water hyacinth plant. After which any of the nutritive medium may be used for the mass production of this pathogenic strain. It should also be noted that water hyacinth leaf agar (WHA) supported the growth of the entire organisms, and that WHA is the cheapest medium used in the study. This also suggests that the mass production of any of the

organisms using the formulated water hyacinth leaf agar (WHA) medium is most economical.

Effect of Nitrogen sources on Growth

In this study, the rate of growth of *C. pallescens* on sodium glutamate and sodium nitrate significantly differs from that on other media types. Both media contain sodium metal, suggesting that sodium metal is a very important element in the assimilation and growth of the organism. It is also evident in this study that *C. pallescens* grew better in sodium glutamate medium as compared with sodium nitrate medium (Czapek Dox). This strongly points to the fact that an organic nitrogen source is needed for the assimilation and growth of *C. pallescens.*

With this study, the rate of growth of *F. solani* on sodium glutamate and sodium nitrate significantly differs from that on other media types. Both media contain sodium metal, suggesting that sodium metal is a very important element in the assimilation of food and growth of the organism. It is also important to note that this organism grew better in sodium nitrate medium (Czapek-Dox) as compared to sodium glutamate containing medium. This suggests that inorganic sodium containing nitrogen source is needed for the assimilation and growth of *F. solani.*

In this study, *M. roridum* (IMI 394934) grew better in sodium glutamate medium as compared with sodium nitrate medium (Czapek Dox). This also points to the fact that an organic nitrogen source is needed for the assimilation nutrient and growth of *M. roridum* (IMI 394934). Ammonium chloride appeared as poor nitrogen source for growth of the fungi in this study and should be excluded when compounding the growth medium for

these organisms. This also indicates that the growth of fungi is specific to the type of nitrogen source present in the medium.

Optimal pH for the Growth of the Organisms

The organisms grew at different rates over the pH range. They all grew best at acidic pH. In this study, the pH range between 6.6 and 8.6 appeared too high for nutrient breakdown and assimilation in *C. pallescens*. The optimal growth pH obtained in this work was approximately the same as that repoted by Sonia *et al.*, (1998) on *C. pallescens*.

Cellulase Activity of Phytopathogens

Cellulase enzymes were produced by the three fungal isolates on the three different carbon sources; carboxymethylcellulose (enzyme substrate grade), sawdust and water hyacinth. The enzyme production was highest with *Curvularia pallescens*, followed by *Fusarium solani* and least with *Myrothecium roridum* (IMI 394934). The cellulose enzyme production by the three organisms was most favoured on the medium containing carboxymethylcellulose as the sole carbon source. This suggests that CMC is a good carbon source for enzyme production by fungal species. Moreso, the hemicellulase enzyme, xylanase is required for the hydrolysis of cellulose in plant cell wall (Khan, 1980). The production of this enzyme by the three isolates was most favoured on CMC. This was more produced when compared to the endoglucanase activity of the three isolates when CMC and sawdust were used as the carbon sources. It was more than the endoglucanase and exoglucanase specific activity of the isolates when water hyacinth was used as the carbon source. Most fungi generally have the potential to depolymerize hemicellulose (xylan) than cellulose due to the solubility of the hemicellulose (Grant and Long, 1981). The production of β-glucosidase was highest by the water hyacinth

pathogenic fungus when compared to the non-pathogenic isolates. The β-glucosidase enzyme production was highest when compared to the endoglucanase, exoglunase, and xylanase enzyme from the same pathogenic isolate. Although, a related species, *Myrothecium verucarria* has been shown to produce a consistent result with β-glucosidase, endoglucanase, exoglunase, and xylanase when different carbon sources were used (Moreira *et al.*, 2005). Extracellular enzymes are important to fungi not only for digestion but also in many instances for the pathogenic process: the enzymes may function in overcoming the natural resistance of the host as well as in providing soluble products that can be absorbed and used as food (Griffin, 1994). Therefore, the enzymes produced by *Myrothecium roridum* (IMI 394934) may be seen as pathogenic factors in the penetration of the plant material rather than the virulence factor since the avirulent isolates were also able to produce a higher amount of these enzymes. In this study, the water hyacinth leaf appeared the poorest carbon source for enzyme production by the three isolates. This suggests that there could be some enzyme inhibitors in the water hyacinth leaf or that the level of cellulose in the water hyacinth leaf was too small for cellulases and hemicellulases induction. Plant proteinases have been implicated in the inhibiton of enzyme production by a plant pathogenic fungus (Moreira *et al.*, 2005).

Phytotoxin Production

This research shows that the isolate of *M. roridum* (IMI 394934) used in this study did not produce phytotoxin activity when cultivated in-vitro 24 hours dark period as judged by the negative results for the bioassays on the leaves of water hyacinth (*E. crassipes*) plant. Under 12 hours light/dark cycle phytotoxin was produced appreciably. Evidence of phytotoxin activity was initially observed as dark spot on the leaves which latter turned

the leaves necrotic at the point of toxin application and further extended both transversely and longitudinally across the whole leaves. This foliar symptom resulting from the phytotoxin activity was similar to those induced by the fungus itself. Similar work has shown that *Alternaria eichhorniae* produced two non-specific phytotoxins of water hyacinth when grown on potato dextrose broth for 3 to 4 weeks under 12 hours photoperiod (Charudattan and Rao, 1982).

Effect of pH on Phytotoxin Production

To ascertain the maximum pH for the production of the toxin by this isolate, a pH range between 4.0 and 7.5 was used. Phytotoxin was produced over a pH range 4.5 to 7.5. pH 4.0 did not support the toxin production and this is probably due to the little or no biomass production at that pH over the fermentation period. The maximum toxin production by this organism was observed at pH 4.5; a second lesser optimum production was at pH 6.0 and 7.0. It is evident from this study that toxigenic fungi produce toxin at a broad pH range but their pH maximum for phytotoxin production are specific. *Fusarium solani* f. sp. *Piperis* in potato sucrose culture medium produced phytotoxin best at an optimum pH 6.0 and also at a second lesser optimum pH 4.5 (Duarte and Archer, 2003). Culture media adjusted to pH 6.7 and even 7.0 are used to promote *Verticillium dahliae* and *Curvularia lunata* toxins respectively (Nachmias *et al.*, 1987; Patel *et al.*, 1987; Duarte and Archer, 2003). The optimum pH for *Corynespora cassicola* toxin production is pH 6 – 7 (Onesirosan *et al.*, 1975; Duarte and Archer, 2003).

Effect of Media on Phytotoxin Production

In order to determine the most cost effective and the best medium for phytotoxin production by *M roridum* (IMI 394934) six defined media types and homogenized water

hyacinth leaf broth (WHB) were used. Sabouraud broth, Czapek-Dox broth or potato sucrose broth produced a significantly lesser amount of phytotoxin when compared to potato carrot broth (PCB) and water hyacinth leaf broth (WHB). Czapek-Dox broth has been reported to induce a lesser production of phytotoxic metabolite when inoculated with *Fusarium solani* (Duarte and Archer, 2003). Potato carrot broth (PCB) and homogenized water hyacinth leaf broth (WHB) optimally induced phytotoxin production, and this is probably because they are plant materials. Some fungal strains could require specific components to induce the synthesis of phytotoxin, such as exist in the complex biochemical make-up of the host plant that determined these fungal strains to be host-specific. For example, plants containing activators such as 2-amino-1,3-propandiol (serinol) produced in sugarcane leaves have been reported to induce phytotoxin production in attenuated strains of *Helminthosporium sacchari* (Pinkerton and Strobel, 1976; Barbosa *et al.*, 2002). This study suggest that water hyacinth contains some components which signal phytotoxin production by *M. roridum* (IMI 394934) and it should also be noted that this medium is the cheapest, which makes it the most economical medium to use in toxin production.

Effect of Carbon Source on Phytotoxin Production

In this study, xylose; a pentose sugar was the best enhancer of phytotoxin production by *M. roridum* (IMI 394934). Although glucose is commonly used as carbon source by most fungi, including those producing phytotoxin, it appeared the fifth best in terms of phytotoxin production in our study. Sugars such as fructose, mannose, galactose, xylose and sucrose have been reported to be good carbon sources, but require an adaptation phase as they must be phosphorylated prior to interconversion during glycolysis (Gadd,

1988; Barbosa *et al.*, 2002). Xylose is metabolized via the pentose phosphate pathway, while sucrose must first be hydrolyzed to its constituent sugars (Barbosa *et al.*, 2002). Fructose and sucrose, along with glucose, are common plant sugars produced by photosynthesis (Raven *et al.*, 1992; Barbosa *et al.*, 2002) they fairly supported phytotoxin production of *M. roridum* (IMI 394934) when compared to xylose sugar in this study. In this work, lactose; a disaccharide sugar was the second best in phytotoxin production.

Effect of Nitrogen Source on Phytotoxin Production

Both organic and inorganic nitrogenous compound were able to enhance phytotoxin in *M. roridum*. Among various nitrogen sources investigated, glutamine appeared the best for toxin production, the second best was monosodium glutamate and the third best was urea. This result suggests that an organic nitrogen source is preferred by *M. roridum* (IMI 394934) in phytotoxin production.

The yeast extract was the least in phytotoxin production in the fungus, although it gave the highest biomass production followed by monosodium glutamate and glutamine. A similar work have shown that after the use of various nitrogen sources in phytotoxin production by *B. euphorbiae*, and a subsequent dilution of the cell free culture extract, the yeast extract grown cultures lost phytotoxin activity, but the cell free extract from other grown culture, nonetheless maintained their biological potency even at dilutions of 1:20 (Barbosa *et al.*, 2002).

Effect of Light Regimes on the Potency of Phytotoxin Activity

In this study, the phytotoxin produced by *M. roridum* (IMI 394934) was biologically active in both the 24 hours dark period and 12 hours light/dark cycle, although, some phytotoxin have been reported to be photosensitive. For example, cercosporin produced

by *Cercospora* sp. was reported to be biologically active only in the light (Spikes, 1989). Many plant pathogenic fungi utilize toxins for successful pathogenesis of their hosts. Among these are the photoactivated perylenequinones, produced by a number of important fungal plant pathogens, including species of *Alternaria*, *Cercospora*, *Cladosporium*, *Elsinoe*, and *Hypocrella*, among others (Daub and Chung, 2007). The advantage of the phytotoxin obtained in this study is that it is bioactive in both the light and dark periods.

Effect of pH on the Potency of Phytotoxin Activity

At the end of the fermentation period, the culture filtrate attained the alkaline pH between 8.1 and 8.7 from an initial pH ranging between 4.5 and 7.5. The phytotoxin of *M. roridum* (IMI 394934) was not active at pH 4.0 and most active at pH 6.0. This suggests that for maximum biological activity of the phytotoxin to be achieved, the pH has to be adjusted to pH 6.0.

The phytotoxin produced by this isolate was active over a pH range 4.5-7.5. Fungi produce phytotoxins that are bioactive at a broad pH range. For example, the phytotoxins of *B. euphorbiae* was active over a broad pH range between 3 and 9 (Barbosa *et al.*, 2002). Similarly, the toxin from *H. nodulosum* was active at pH range 3-7, but inactive within the alkaline range (Barbosa *et al.*, 2002).

Effect of Heat on the Potency of Phytotoxin Activity

The detached leaves of water hyacinth (*Eichhornia crassipes*) showed necrotic symptoms at the point of infiltration of both autoclaved and non-autoclaved *M. roridum* (IMI 394934) culture filtrate, indicating the thermostable nature of this metabolite. The autoclaved toxin showed more disease severity on the leaves when compared with the

non-autoclaved culture filtrate. This could be probably due to the destruction of some metabolites in the crude toxin which acts as antagonists to the active compound. Thermostability has been observed for many host-specific and non-host specific toxins (Duarte and Archer, 2003). Metha and Brogin (2002) reported that culture filtrate produced by *Stemphylium solani* Weber was shown to be stable during autoclaving.

Biological activity of phytotoxin on leaves of various plants.

The phtyotoxin produced by this fungus affected the leaves of four out of twenty six types of plants tested. Although, the ASI of the affected plants were significantly lower than ASI of water hyacinth. This result suggests that the toxin is host non-specific as agaist the fungus which appeared to be host specific. However, the discovery that four economic crops are susceptible to the phytotoxin indicates that caution is required in the application of this potential herbicide.

Isolation and Partial Characterization of Phytotoxin

The extraction of phytotoxins from fungal culture with organic solvents yielded a yellowish-brown liquid, which when lyophilzed, formed a greasy, yellow crystalline product. After purification on TLC plate the pure phytotoxic fraction formed a white crystalline product, this appeared colourless in liquid solutions and was detected on TLC plate using vanillin sulphuric acid spray. These properties conform to that of trichothecenes described in literatures (Cole and Cox, 1981; National Research Council, 1983). Trichothecenes have been reported to be produced by *Myrothecium roridum* (Bean *et al.*, 1984). It is probable that the virulence factor responsible for the pathogenicity of the the locally sourced isolate of *M. roridum* (IMI 394934) on water hyacinth is a trichothecene compound.

CHAPTER SIX

6.0 CONCLUSION AND RECOMMENDATION

This study for the first time has generated a documented evidence that the fungal agent "*Myrothecium roridum*" can be sourced locally in Badagry creeks in Lagos and Ogun river in Isheri, Ogun State, Nigeria and that it posseses the level of virulence needed for it to be considered as a potential biocontrol agent for water hyacinth. Also, it is evident in this study that the lethal effect of this isolate on water hyacinth rests on its ability to produce a phytotoxin that is probably a trichothecene derivative. It is also concluded in this study that the phytotoxin from this strain of *M. roridum* can independently kill water hyacinth plant and can be packaged and used as a chemical herbicide in the countries where the fungal pathogen can be affected by the biotic and abiotic conditions. It was discovered that a formulated low cost water hyacinth leaf agar medium can be used for the mass production of the isolate after the pure culture has been obtained using tap water agar medium. Finally, the same formulated low cost water hyacinth leaf broth can be used to grow this isolate for phytotoxin production. In this study, I isolated and identified a highly virulent strain of *M. roridum* which is potentially useful as a biocontrol agent or source of herbicide (phytotoxin) for water hyacinth (*Eichhornia crassipes*) control.

I therefore recommend that further research work should be carried out to elucidate the chemical structure of the specific metabolite implicated as the virulence factor in *M. roridum*.

CONTRIBUTIONS TO KNOWLEDGE

1) In this study, an indigenous strain of a fungus, *M. roridum,* which has potential application as a biocontrol agent against water hyacinth (*Eichhornia crassipes*) was identified.
2) The virulence of the isolated strain was higher than that previously reported for any strain of *M. roridum.*
3) The strain of *M. roridum* and the DNA sequence of its internal transcribed spacer (ITS4) has been deposited and registered with the International Mycological Institute (now called: Center for Agriculture and Bioscience International, Egham, Surrey, United Kingdom) and the organism has been given the accession number: IMI 394934.
4) A heat stable phytotoxin produced by *M. roridum* has been identified as the virulence factor.
5) The phytotoxin has been partially characterized and it is likely to be a trichothecene.

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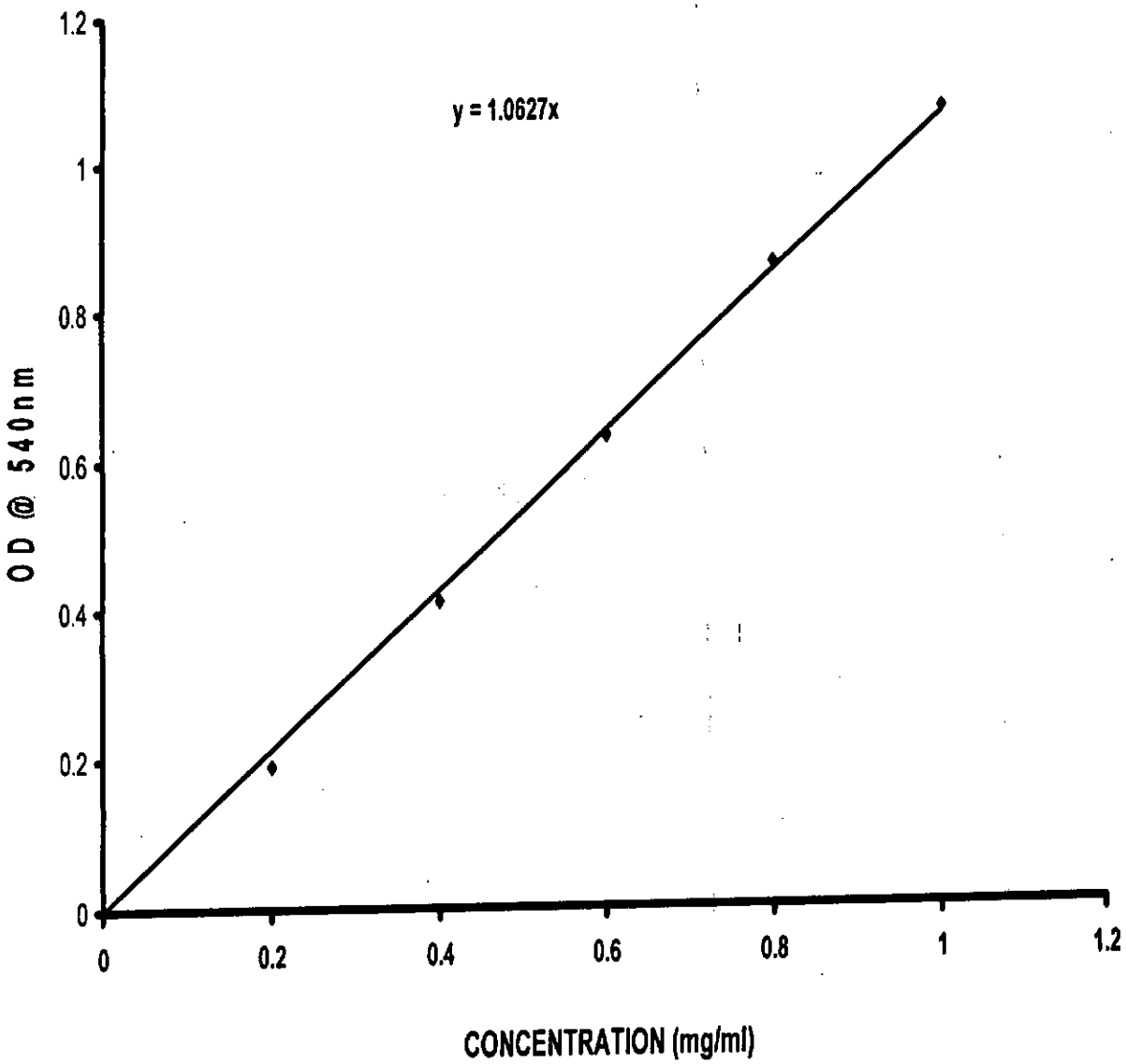
APPENDICES

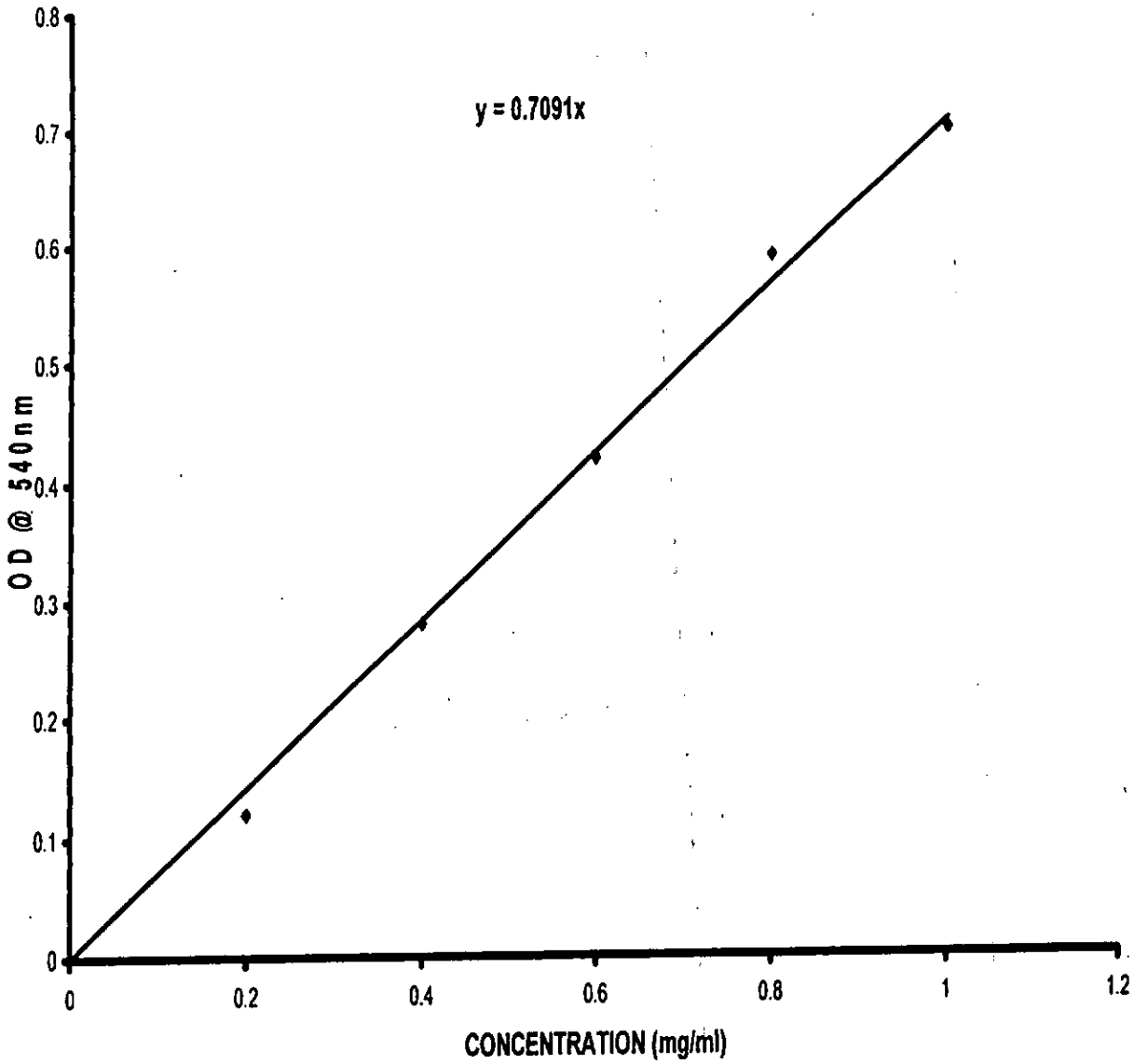
(A)

GLUCOSE STANDARD CURVE



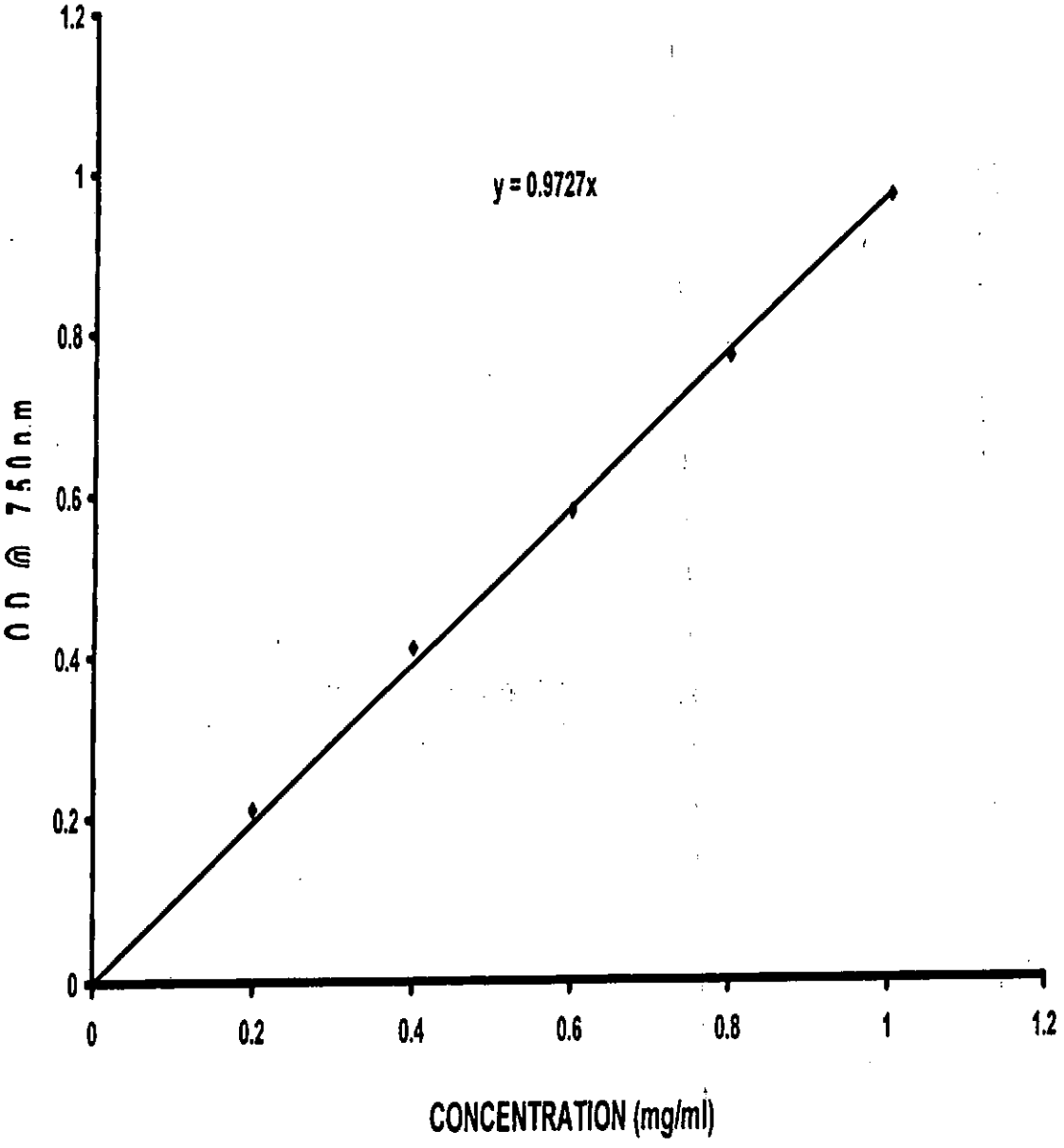
(B)

XYLOSE STANDARD CURVE



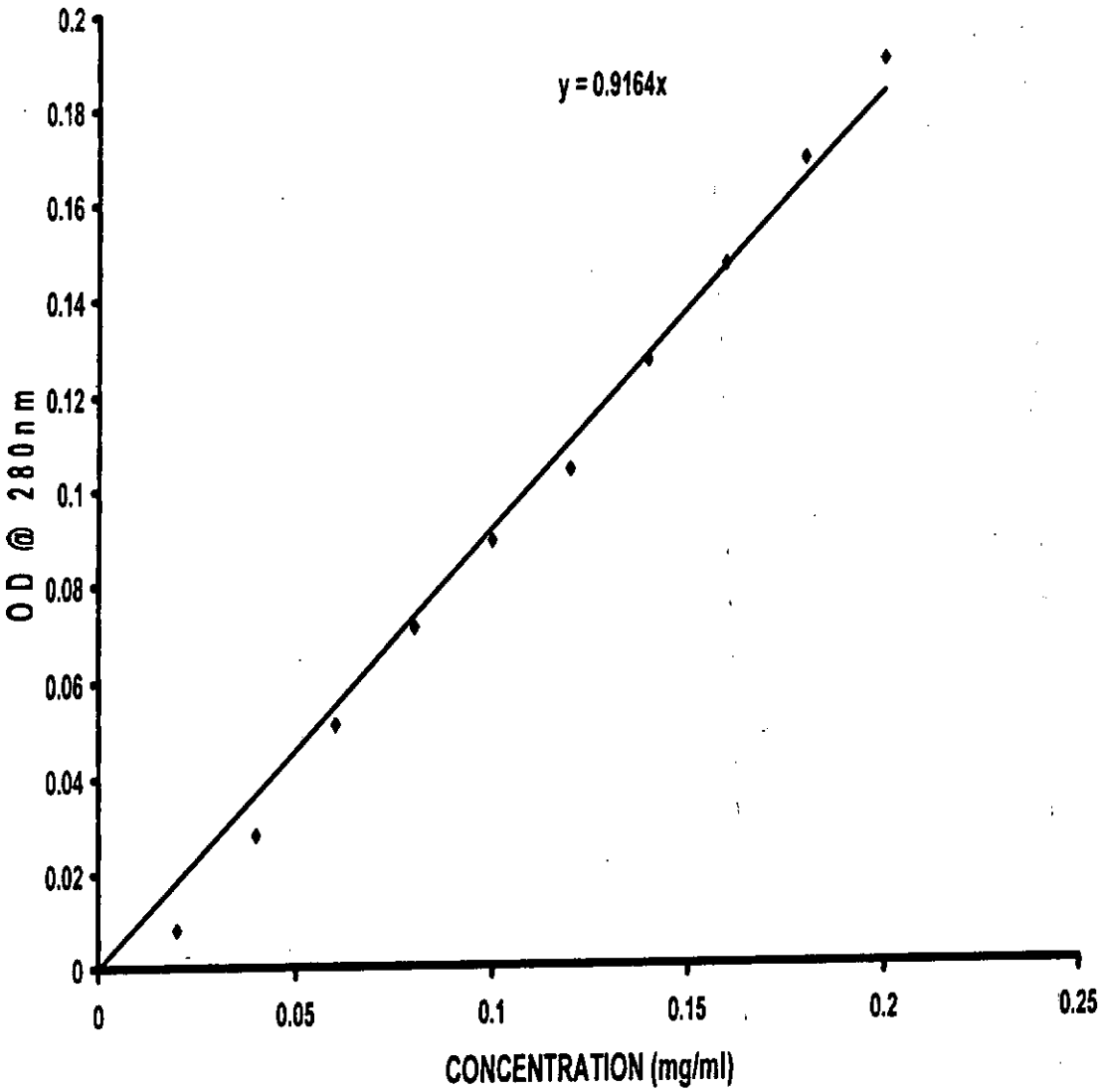
(C)

PROTEIN STANDARD CURVE(BSA)



(D)

PROTEIN STD CURVE (EGG ALBUMIN)



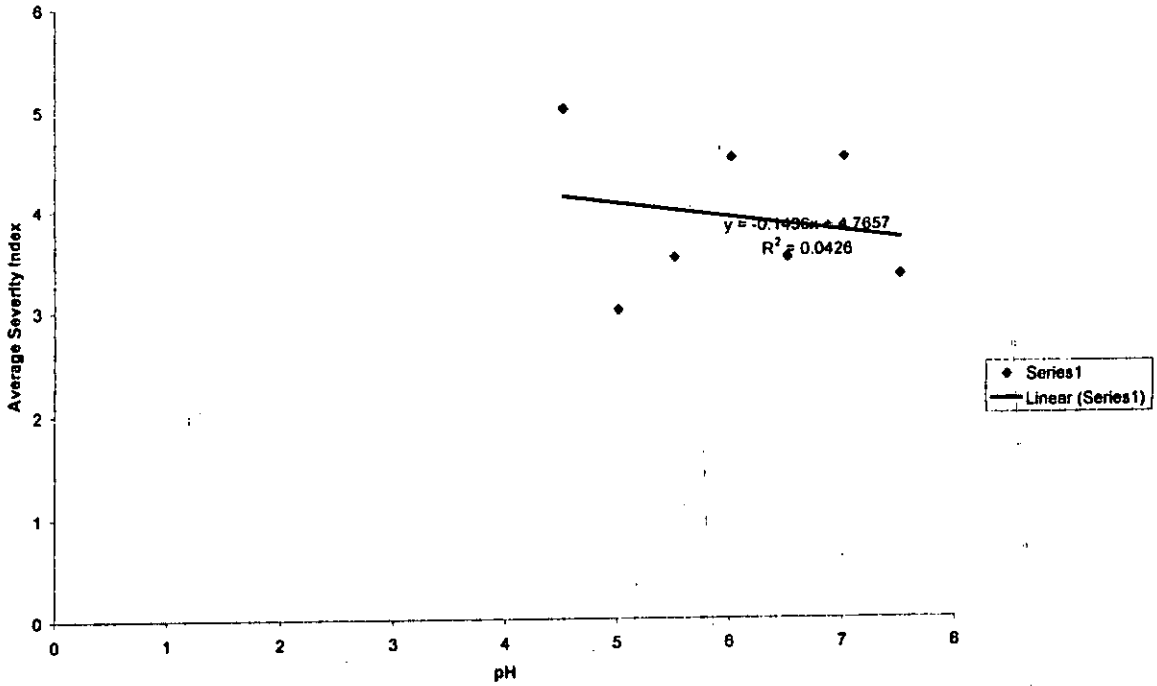
Standard curves:

(A) Glucose standard curve, Miller method (O.D. @ 540 nm)

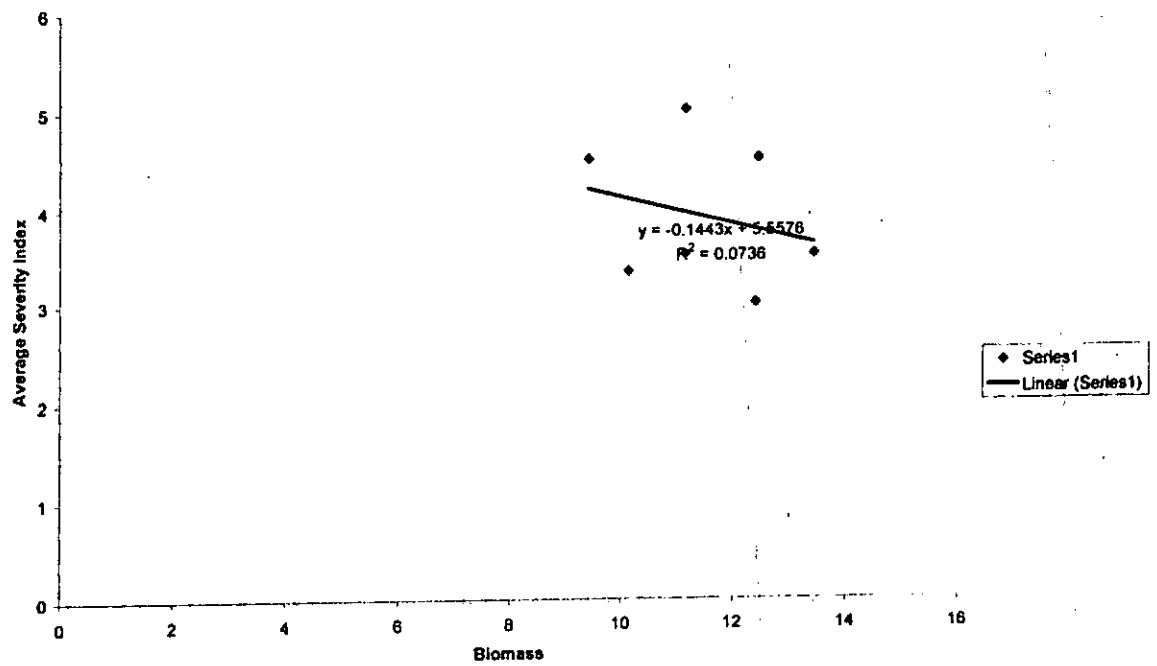
(B) Xylose standerd curve, Miller method (O. D. @ 540 nm)

(C) Protein standard curve, Lowry method (O.D. @ 750 nm)

(D) Protein standard curve, (By extinction @ 280 nm



Relationship Between Biological activity and pH of of Phytotoxin Production by *M. roridum* (IMI 394934)



Relationship Between Biological activity and Biomass Production by M. roridum (IMI 394934)

Table 4.11: R_f Values of Metabolites Produced by *Myrothecium roridum* (IMI 394934) in Different Resolving Solvents (Ethylacetate: methanol and Ethylacetate: methanol: water: acetic acid).

Ethylacetate: methanol (9:1)	Ethylacetate: methanol: water: acetic acid (10:1.7:1.3:0.2)	Characteristics
0.953	0.68	Purple
0.53	0.58	Yellow
0.41	0.41	Light yellow
0.09	0.10	Brown

Table 4.12: R_f Values of *Myrothecium roridum* (IMI 394934) Toxin Eluted under Vacuum Chromatography with Ethylacetate: Methanol: Water: Acetic Acid as Resolving Solvent.

Eluent 1	Characteristics	Eluent 2	Characteristics	Eluent 3	Characteristics
0.83	Vanillin +	0.91	UV +	0.91	UV +
0.69	Vanillin +	0.83	Fluoresce, Vanillin +	0.83	UV +
		0.66	UV +	0.66	UV +
		0.61	UV +	0.61	UV +
		0.55	UV +	0.55	UV +
		0.30	Fluoresce	0.45	UV +
		0.19	Fluoresce	0.39	UV +
		0.08	Fluoresce		

Table 4.13: R_f Values of *Myrothecium roridum* (IMI 394934) Toxin Eluted under Vacuum Chromatography with Methanol: Dichloromethane (8:92) as Resolving Solvent

Eluent 1	Characteristics	Eluent 2	Characteristics	Eluent 3	Characteristics
0.94	UV +, Vanillin +	0.94	UV +	0.51	Vanillin +
0.87	UV +	0.75	UV +	0.45	UV +
0.75	UV +	0.54	Colour	0.30	UV +
0.51	Vanillin +	0.51	Vanillin +	0.23	Vanillin +
0.43	UV +	0.45	UV +	0.21	Vanillin +
		0.42	Colour	0.18	Vanillin +
		0.37	UV +		
		0.30	Vanillin +		
		0.27	UV +		
		0.22	Colour, Vanillin +		
		0.17	Colour, Vanillin		
		0.12	Colour		
		0.05	Colour		

Table 4.14: R_f Values of *Myrothecium roridum* (IMI 394934) Toxin Eluted under Vacuum Chromatography with Methanol: Dichloromethane: Hexane (4:92:4) as Resolving Solvent

Eluent 1	Characteristics	Eluent 2	Characteristics	Eluent 3	Characteristics
0.92	UV +	0.93	UV +	0.54	Vanillin +
0.89	UV +	0.72	colour	0.48	UV +
0.71	UV +	0.53	colour	0.43	UV +
0.53	Vanillin +	0.49	UV +	0.40	Vanillin +
0.46	UV +	0.47	colour	0.33	Vanillin +
		0.42	UV +	0.29	Vanillin +
		0.39	Colour, UV +, Vanillin	0.24	Vanillin +
		0.34	Colour		
		0.31	Vanillin		
		0.27	Colour		
		0.12	colour		

Relationship Between pH, Biomass and Biological Activity of Crude Toxin Produced

Correlations

		pH	Biomass	Biological Activity
pH	Pearson Correlation	1	-.622	-.206
	Sig. (2-tailed)	.	.136	.657
	N	7	7	7
Biomass	Pearson Correlation	-.622	1	-.271
	Sig. (2-tailed)	.136	.	.556
	N	7	7	7
Biological Activity	Pearson Correlation	-.206	-.271	1
	Sig. (2-tailed)	.657	.556	.
	N	7	7	7

T-Test. Comparation of Concentration Difference for Unautoclaved crude toxin produce in media types. Where 1=100%, 2=70%, 3=50%, 4=10%, 5=1%.

Group Statistics

	Concn	N	Mean	Std. Deviation	Std. Error Mean
MAB	1.00	6	.0000	.00000	.00000
	2.00	6	2.6667	.81650	.33333
PCB	1.00	6	3.3333	.51640	.21082
	2.00	6	2.6667	.51640	.21082
PDB	1.00	6	.6667	.51640	.21082
	2.00	6	3.0000	.63246	.25820
PSB	1.00	6	1.0000	.63246	.25820
	2.00	6	1.3333	.51640	.21082
WHB	1.00	6	1.6667	.51640	.21082
	2.00	6	3.0000	.63246	.25820
SB	1.00	6	.0000	.00000	.00000
	2.00	6	2.6667	.51640	.21082
Z-DOX	1.00	6	.0000	.00000	.00000
	2.00	6	1.6667	.81650	.33333

Independent Samples Test

		Levene's Test for Equality of Variances		t-test for Equality of Means						
									95% Confidence Interval of the Difference	
		F	Sig.	t	df	Sig. (2-tailed)	Mean Difference	Std. Error Difference	Lower	Upper
MAB	Equal variances assumed	20.000	.001	-8.000	10	.000	-2.6667	.33333	-3.40938	-1.92395
	Equal variances not assumed			-8.000	5.000	.000	-2.6667	.33333	-3.52353	-1.80981
PCB	Equal variances assumed	.000	1.000	2.236	10	.049	.6667	.29814	.00236	1.33097
	Equal variances not assumed			2.236	10.000	.049	.6667	.29814	.00236	1.33097
PDB	Equal variances assumed	.250	.628	-7.000	10	.000	-2.3333	.33333	-3.07605	-1.59062
	Equal variances not assumed			-7.000	9.615	.000	-2.3333	.33333	-3.08009	-1.58657
PSB	Equal variances assumed	.250	.628	-1.000	10	.341	-.3333	.33333	-1.07605	.40938
	Equal variances not assumed			-1.000	9.615	.342	-.3333	.33333	-1.08009	.41343
WHB	Equal variances assumed	.250	.628	-4.000	10	.003	-1.3333	.33333	-2.07605	-.59062
	Equal variances not assumed			-4.000	9.615	.003	-1.3333	.33333	-2.08009	-.58657
SB	Equal variances assumed	40.000	.000	-12.649	10	.000	-2.6667	.21082	-3.13640	-2.19693
	Equal variances not assumed			-12.649	5.000	.000	-2.6667	.21082	-3.20859	-2.12474
Z-DOX	Equal variances assumed	20.000	.001	-5.000	10	.001	-1.6667	.33333	-2.40938	-.92395
	Equal variances not assumed			-5.000	5.000	.004	-1.6667	.33333	-2.52353	-.80981

Group Statistics

	Concn	N	Mean	Std. Deviation	Std. Error Mean
MAB	1.00	6	.0000	.00000	.00000
	3.00	6	2.3333	.81650	.33333
PCB	1.00	6	3.3333	.51640	.21082
	3.00	6	.0000	.00000	.00000
PDB	1.00	6	.6667	.51640	.21082
	3.00	6	.0000	.00000	.00000
PSB	1.00	6	1.0000	.63246	.25820
	3.00	6	.0000	.00000	.00000
WHB	1.00	6	1.6667	.51640	.21082
	3.00	6	3.3333	.81650	.33333
SB	1.00	6	.0000	.00000	.00000
	3.00	6	1.3333	.51640	.21082
Z-DOX	1.00	6	.0000	.00000	.00000
	3.00	6	.3333	.51640	.21082

Independent Samples Test

		Levene's Test for Equality of Variances		t-test for Equality of Means						
									95% Confidence Interval of the Difference	
		F	Sig.	t	df	Sig. (2-tailed)	Mean Difference	Std. Error Difference	Lower	Upper
MAB	Equal variances assumed	20.000	.001	-7.000	10	.000	-2.3333	.33333	-3.07605	-1.59062
	Equal variances not assumed			-7.000	5.000	.001	-2.3333	.33333	-3.19019	-1.47647
PCB	Equal variances assumed	40.000	.000	15.811	10	.000	3.3333	.21082	2.86360	3.80307
	Equal variances not assumed			15.811	5.000	.000	3.3333	.21082	2.79141	3.87526
PDB	Equal variances assumed	40.000	.000	3.162	10	.010	.6667	.21082	.19693	1.13640
	Equal variances not assumed			3.162	5.000	.025	.6667	.21082	.12474	1.20859
PSB	Equal variances assumed	2.500	.145	3.873	10	.003	1.0000	.25820	.42470	1.57530
	Equal variances not assumed			3.873	5.000	.012	1.0000	.25820	.33628	1.66372
WHB	Equal variances assumed	1.818	.207	-4.226	10	.002	-1.6667	.39441	-2.54546	-.78788
	Equal variances not assumed			-4.226	8.448	.003	-1.6667	.39441	-2.56783	-.76550
SB	Equal variances assumed	40.000	.000	-6.325	10	.000	-1.3333	.21082	-1.80307	-.86360
	Equal variances not assumed			-6.325	5.000	.001	-1.3333	.21082	-1.87526	-.79141
Z-DOX	Equal variances assumed	40.000	.000	-1.581	10	.145	-.3333	.21082	-.80307	.13640
	Equal variances not assumed			-1.581	5.000	.175	-.3333	.21082	-.87526	.20859

Group Statistics

	Concn	N	Mean	Std. Deviation	Std. Error Mean
MAB	1.00	6	.0000	.00000	.00000
	4.00	6	3.0000	.89443	.36515
PCB	1.00	6	3.3333	.51640	.21082
	4.00	6	.0000	.00000	.00000
PDB	1.00	6	.6667	.51640	.21082
	4.00	6	.3333	.51640	.21082
PSB	1.00	6	1.0000	.63246	.25820
	4.00	6	.0000	.00000	.00000
WHB	1.00	6	1.6667	.51640	.21082
	4.00	6	2.6667	.51640	.21082
SB	1.00	6	.0000	.00000	.00000
	4.00	6	1.0000	.63246	.25820
Z-DOX	1.00	6	.0000	.00000[a]	.00000
	4.00	6	.0000	.00000[a]	.00000

a. t cannot be computed because the standard deviations of both groups are 0.

Independent Samples Test

		Levene's Test for Equality of Variances		t-test for Equality of Means						
									95% Confidence Interval of the Difference	
		F	Sig.	t	df	Sig. (2-tailed)	Mean Difference	Std. Error Difference	Lower	Upper
MAB	Equal variances assumed	10.000	.010	-8.216	10	.000	-3.0000	.36515	-3.81360	-2.18640
	Equal variances not assumed			-8.216	5.000	.000	-3.0000	.36515	-3.93864	-2.06136
PCB	Equal variances assumed	40.000	.000	15.811	10	.000	3.3333	.21082	2.86360	3.80307
	Equal variances not assumed			15.811	5.000	.000	3.3333	.21082	2.79141	3.87526
PDB	Equal variances assumed	.000	1.000	1.118	10	.290	.3333	.29814	-.33097	.99764
	Equal variances not assumed			1.118	10.000	.290	.3333	.29814	-.33097	.99764
PSB	Equal variances assumed	2.500	.145	3.873	10	.003	1.0000	.25820	.42470	1.57530
	Equal variances not assumed			3.873	5.000	.012	1.0000	.25820	.33628	1.66372
WHB	Equal variances assumed	.000	1.000	-3.354	10	.007	-1.0000	.29814	-1.66430	-.33570
	Equal variances not assumed			-3.354	10.000	.007	-1.0000	.29814	-1.66430	-.33570
SB	Equal variances assumed	2.500	.145	-3.873	10	.003	-1.0000	.25820	-1.57530	-.42470
	Equal variances not assumed			-3.873	5.000	.012	-1.0000	.25820	-1.66372	-.33628

T-Test. Effect of Autoclaving Crude Toxin on Biological Activity. Where 1=100%, 2=70%, 3=50%, 4=10%, 5=1%.

Group Statistics

	Concn	N	Mean	Std. Deviation	Std. Error Mean
MAB	1.00	6	.0000	.00000	.00000
	5.00	6	2.6667	.81650	.33333
PCB	1.00	6	3.3333	.51640	.21082
	5.00	6	2.0000	.63246	.25820
PDB	1.00	6	.6667	.51640	.21082
	5.00	6	2.3333	.51640	.21082
PSB	1.00	6	1.0000	.63246	.25820
	5.00	6	1.6667	.51640	.21082
WHB	1.00	6	1.6667	.51640	.21082
	5.00	6	1.6667	.51640	.21082
SB	1.00	6	.0000	.00000	.00000
	5.00	6	2.0000	.63246	.25820
Z-DOX	1.00	6	.0000	.00000	.00000
	5.00	6	2.3333	.81650	.33333

Independent Samples Test

		Levene's Test for Equality of Variances		t-test for Equality of Means						
									95% Confidence Interval of the Difference	
		F	Sig.	t	df	Sig. (2-tailed)	Mean Difference	Std. Error Difference	Lower	Upper
MAB	Equal variances assumed	20.000	.001	-8.000	10	.000	-2.6667	.33333	-3.40938	-1.92395
	Equal variances not assumed			-8.000	5.000	.000	-2.6667	.33333	-3.52353	-1.80981
PCB	Equal variances assumed	.250	.628	4.000	10	.003	1.3333	.33333	.59062	2.07605
	Equal variances not assumed			4.000	9.615	.003	1.3333	.33333	.58657	2.08009
PDB	Equal variances assumed	.000	1.000	-5.590	10	.000	-1.6667	.29814	-2.33097	-1.00236
	Equal variances not assumed			-5.590	10.000	.000	-1.6667	.29814	-2.33097	-1.00236
PSB	Equal variances assumed	.250	.628	-2.000	10	.073	-.6667	.33333	-1.40938	.07605
	Equal variances not assumed			-2.000	9.615	.075	-.6667	.33333	-1.41343	.08009
WHB	Equal variances assumed	.000	1.000	.000	10	1.000	.0000	.29814	-.66430	.66430
	Equal variances not assumed			.000	10.000	1.000	.0000	.29814	-.66430	.66430
SB	Equal variances assumed	2.500	.145	-7.746	10	.000	-2.0000	.25820	-2.57530	-1.42470
	Equal variances not assumed			-7.746	5.000	.001	-2.0000	.25820	-2.66372	-1.33628
Z-DOX	Equal variances assumed	20.000	.001	-7.000	10	.000	-2.3333	.33333	-3.07605	-1.59062
	Equal variances not assumed			-7.000	5.000	.001	-2.3333	.33333	-3.19019	-1.47647

Group Statistics

	Concn	N	Mean	Std. Deviation	Std. Error Mean
MAB + Autoclaving	1.00	6	2.3333	.81650	.33333
	2.00	6	4.3333	.51640	.21082
PCB + Autoclaving	1.00	6	3.0000	.63246	.25820
	2.00	6	3.6667	.81650	.33333
PDB + Autoclaving	1.00	6	1.0000	.63246	.25820
	2.00	6	4.6667	.51640	.21082
PSB + Autoclaving	1.00	6	1.3333	.51640	.21082
	2.00	6	2.3333	.51640	.21082
WHB + Autoclaving	1.00	6	2.6667	.51640	.21082
	2.00	6	3.3333	.81650	.33333
SB + Autoclaving	1.00	6	1.0000	.63246	.25820
	2.00	6	4.0000	.63246	.25820
Z-DOX + Autoclaving	1.00	6	1.3333	.51640	.21082
	2.00	6	1.6667	.51640	.21082

Independent Samples Test

		Levene's Test for Equality of Variances		t-test for Equality of Means						
									95% Confidence Interval of the Difference	
		F	Sig.	t	df	Sig. (2-tailed)	Mean Difference	Std. Error Difference	Lower	Upper
MAB + Autoclaving	Equal variances assumed	1.818	.207	-5.071	10	.000	-2.0000	.39441	-2.87879	-1.12121
	Equal variances not assumed			-5.071	8.448	.001	-2.0000	.39441	-2.90117	-1.09883
PCB + Autoclaving	Equal variances assumed	1.667	.226	-1.581	10	.145	-.6667	.42164	-1.60613	.27280
	Equal variances not assumed			-1.581	9.412	.147	-.6667	.42164	-1.61415	.28082
PDB + Autoclaving	Equal variances assumed	.250	.628	-11.000	10	.000	-3.6667	.33333	-4.40938	-2.92395
	Equal variances not assumed			-11.000	9.615	.000	-3.6667	.33333	-4.41343	-2.91991
PSB + Autoclaving	Equal variances assumed	.000	1.000	-3.354	10	.007	-1.0000	.29814	-1.66430	-.33570
	Equal variances not assumed			-3.354	10.000	.007	-1.0000	.29814	-1.66430	-.33570
WHB + Autoclaving	Equal variances assumed	1.818	.207	-1.690	10	.122	-.6667	.39441	-1.54546	.21212
	Equal variances not assumed			-1.690	8.448	.127	-.6667	.39441	-1.56783	.23450
SB + Autoclaving	Equal variances assumed	.000	1.000	-8.216	10	.000	-3.0000	.36515	-3.81360	-2.18640
	Equal variances not assumed			-8.216	10.000	.000	-3.0000	.36515	-3.81360	-2.18640
Z-DOX + Autoclaving	Equal variances assumed	.000	1.000	-1.118	10	.290	-.3333	.29814	-.99764	.33097
	Equal variances not assumed			-1.118	10.000	.290	-.3333	.29814	-.99764	.33097

Group Statistics

	Concn	N	Mean	Std. Deviation	Std. Error Mean
MAB + Autoclaving	1.00	6	2.3333	.81650	.33333
	3.00	6	1.3333	.51640	.21082
PCB + Autoclaving	1.00	6	3.0000	.63246	.25820
	3.00	6	1.3333	.51640	.21082
PDB + Autoclaving	1.00	6	1.0000	.63246	.25820
	3.00	6	.0000	.00000	.00000
PSB + Autoclaving	1.00	6	1.3333	.51640	.21082
	3.00	6	2.3333	.51640	.21082
WHB + Autoclaving	1.00	6	2.6667	.51640	.21082
	3.00	6	4.0000	.89443	.36515
SB + Autoclaving	1.00	6	1.0000	.63246	.25820
	3.00	6	.0000	.00000	.00000
Z-DOX + Autoclaving	1.00	6	1.3333	.51640	.21082
	3.00	6	1.0000	.63246	.25820

Independent Samples Test

		Levene's Test for Equality of Variances		t-test for Equality of Means						
									95% Confidence Interval of the Difference	
		F	Sig.	t	df	Sig. (2-tailed)	Mean Difference	Std. Error Difference	Lower	Upper
MAB + Autoclaving	Equal variances assumed	1.818	.207	2.535	10	.030	1.0000	.39441	.12121	1.87879
	Equal variances not assumed			2.535	8.448	.034	1.0000	.39441	.09883	1.90117
PCB + Autoclaving	Equal variances assumed	.250	.628	5.000	10	.001	1.6667	.33333	.92395	2.40938
	Equal variances not assumed			5.000	9.615	.001	1.6667	.33333	.91991	2.41343
PDB + Autoclaving	Equal variances assumed	2.500	.145	3.873	10	.003	1.0000	.25820	.42470	1.57530
	Equal variances not assumed			3.873	5.000	.012	1.0000	.25820	.33628	1.66372
PSB + Autoclaving	Equal variances assumed	.000	1.000	-3.354	10	.007	-1.0000	.29814	-1.66430	-.33570
	Equal variances not assumed			-3.354	10.000	.007	-1.0000	.29814	-1.66430	-.33570
WHB + Autoclaving	Equal variances assumed	1.000	.341	-3.162	10	.010	-1.3333	.42164	-2.27280	-.39387
	Equal variances not assumed			-3.162	8.000	.013	-1.3333	.42164	-2.30563	-.36104
SB + Autoclaving	Equal variances assumed	2.500	.145	3.873	10	.003	1.0000	.25820	.42470	1.57530
	Equal variances not assumed			3.873	5.000	.012	1.0000	.25820	.33628	1.66372
Z-DOX + Autoclaving	Equal variances assumed	.250	.628	1.000	10	.341	.3333	.33333	-.40938	1.07605
	Equal variances not assumed			1.000	9.615	.342	.3333	.33333	-.41343	1.08009

Group Statistics

	Concn	N	Mean	Std. Deviation	Std. Error Mean
MAB + Autoclaving	1.00	6	2.3333	.81650	.33333
	4.00	6	.6667	.51640	.21082
PCB + Autoclaving	1.00	6	3.0000	.63246	.25820
	4.00	6	4.6667	.51640	.21082
PDB + Autoclaving	1.00	6	1.0000	.63246	.25820
	4.00	6	.3333	.51640	.21082
PSB + Autoclaving	1.00	6	1.3333	.51640	.21082
	4.00	6	.0000	.00000	.00000
WHB + Autoclaving	1.00	6	2.6667	.51640	.21082
	4.00	6	1.6667	.81650	.33333
SB + Autoclaving	1.00	6	1.0000	.63246	.25820
	4.00	6	.0000	.00000	.00000
Z-DOX + Autoclaving	1.00	6	1.3333	.51640	.21082
	4.00	6	.3333	.51640	.21082

Independent Samples Test

		Levene's Test for Equality of Variances		t-test for Equality of Means						
									95% Confidence Interval of the Difference	
		F	Sig.	t	df	Sig. (2-tailed)	Mean Difference	Std. Error Difference	Lower	Upper
MAB + Autoclaving	Equal variances assumed	1.818	.207	4.226	10	.002	1.6667	.39441	.78788	2.54546
	Equal variances not assumed			4.226	8.448	.003	1.6667	.39441	.76550	2.56783
PCB + Autoclaving	Equal variances assumed	.250	.628	-5.000	10	.001	-1.6667	.33333	-2.40938	-.92395
	Equal variances not assumed			-5.000	9.615	.001	-1.6667	.33333	-2.41343	-.91991
PDB + Autoclaving	Equal variances assumed	.250	.628	2.000	10	.073	.6667	.33333	-.07605	1.40938
	Equal variances not assumed			2.000	9.615	.075	.6667	.33333	-.08009	1.41343
PSB + Autoclaving	Equal variances assumed	40.000	.000	6.325	10	.000	1.3333	.21082	.86360	1.80307
	Equal variances not assumed			6.325	5.000	.001	1.3333	.21082	.79141	1.87526
WHB + Autoclaving	Equal variances assumed	1.818	.207	2.535	10	.030	1.0000	.39441	.12121	1.87879
	Equal variances not assumed			2.535	8.448	.034	1.0000	.39441	.09883	1.90117
SB + Autoclaving	Equal variances assumed	2.500	.145	3.873	10	.003	1.0000	.25820	.42470	1.57530
	Equal variances not assumed			3.873	5.000	.012	1.0000	.25820	.33628	1.66372
Z-DOX + Autoclaving	Equal variances assumed	.000	1.000	3.354	10	.007	1.0000	.29814	.33570	1.66430
	Equal variances not assumed			3.354	10.000	.007	1.0000	.29814	.33570	1.66430

Group Statistics

	Concn	N	Mean	Std. Deviation	Std. Error Mean
MAB + Autoclaving	1.00	6	2.3333	.81650	.33333
	5.00	6	3.6667	.81650	.33333
PCB + Autoclaving	1.00	6	3.0000	.63246	.25820
	5.00	6	2.0000	.63246	.25820
PDB + Autoclaving	1.00	6	1.0000	.63246	.25820
	5.00	6	3.6667	.81650	.33333
PSB + Autoclaving	1.00	6	1.3333	.51640	.21082
	5.00	6	2.3333	.81650	.33333
WHB + Autoclaving	1.00	6	2.6667	.51640	.21082
	5.00	6	2.3333	1.03280	.42164
SB + Autoclaving	1.00	6	1.0000	.63246	.25820
	5.00	6	3.6667	.81650	.33333
Z-DOX + Autoclaving	1.00	6	1.3333	.51640	.21082
	5.00	6	3.3333	.81650	.33333

Independent Samples Test

		Levene's Test for Equality of Variances		t-test for Equality of Means						
									95% Confidence Interval of the Difference	
		F	Sig.	t	df	Sig. (2-tailed)	Mean Difference	Std. Error Difference	Lower	Upper
MAB + Autoclaving	Equal variances assumed	.000	1.000	-2.828	10	.018	-1.3333	.47140	-2.38369	-.28298
	Equal variances not assumed			-2.828	10.000	.018	-1.3333	.47140	-2.38369	-.28298
PCB + Autoclaving	Equal variances assumed	.000	1.000	2.739	10	.021	1.0000	.36515	.18640	1.81360
	Equal variances not assumed			2.739	10.000	.021	1.0000	.36515	.18640	1.81360
PDB + Autoclaving	Equal variances assumed	1.667	.228	-6.325	10	.000	-2.6667	.42164	-3.60613	-1.72720
	Equal variances not assumed			-6.325	9.412	.000	-2.6667	.42164	-3.61415	-1.71918
PSB + Autoclaving	Equal variances assumed	1.818	.207	-2.535	10	.030	-1.0000	.39441	-1.87879	-.12121
	Equal variances not assumed			-2.535	8.448	.034	-1.0000	.39441	-1.90117	-.09883
WHB + Autoclaving	Equal variances assumed	1.800	.209	.707	10	.496	.3333	.47140	-.71702	1.38369
	Equal variances not assumed			.707	7.353	.501	.3333	.47140	-.77061	1.43727
SB + Autoclaving	Equal variances assumed	1.667	.226	-6.325	10	.000	-2.6667	.42164	-3.60613	-1.72720
	Equal variances not assumed			-6.325	9.412	.000	-2.6667	.42164	-3.61415	-1.71918
Z-DOX + Autoclaving	Equal variances assumed	1.818	.207	-5.071	10	.000	-2.0000	.39441	-2.87879	-1.12121
	Equal variances not assumed			-5.071	8.448	.001	-2.0000	.39441	-2.90117	-1.09883

T-Test. Effect of pH Media on Toxin Production. Where Concn.: 1=100%, 2=70%, 3=50%, 4=30%, 5=10%, 6=1%.

Group Statistics

	Concn.	N	Mean	Std. Deviation	Std. Error Mean
pH 4.5	1.00	6	5.0000	.00000	.00000
	2.00	6	2.3333	.51640	.21082
pH 5	1.00	6	3.0000	.63246	.25820
	2.00	6	2.3333	.81650	.33333
pH 5.5	1.00	6	3.5000	.54772	.22361
	2.00	6	2.0000	.63246	.25820
pH 6	1.00	6	4.5000	.54772	.22361
	2.00	6	3.0000	.63246	.25820
pH 6.5	1.00	6	.0000	.00000	.00000
	2.00	6	2.6667	.81650	.33333
pH 7	1.00	6	4.5000	.54772	.22361
	2.00	6	2.0000	.89443	.36515
pH 7.5	1.00	6	3.0000	.63246	.25820
	2.00	6	2.6667	.51640	.21082

Independent Samples Test

		Levene's Test for Equality of Variances		t-test for Equality of Means						
									95% Confidence Interval of the Difference	
		F	Sig.	t	df	Sig. (2-tailed)	Mean Difference	Std. Error Difference	Lower	Upper
pH 4.5	Equal variances assumed	40.000	.000	12.649	10	.000	2.6667	.21082	2.19693	3.13640
	Equal variances not assumed			12.649	5.000	.000	2.6667	.21082	2.12474	3.20859
pH 5	Equal variances assumed	1.667	.226	1.581	10	.145	.6667	.42164	-.27280	1.60613
	Equal variances not assumed			1.581	9.412	.147	.6667	.42164	-.28082	1.61415
pH 5.5	Equal variances assumed	.625	.448	4.392	10	.001	1.5000	.34157	.73895	2.26105
	Equal variances not assumed			4.392	9.800	.001	1.5000	.34157	.73684	2.26316
pH 6	Equal variances assumed	.625	.448	4.392	10	.001	1.5000	.34157	.73895	2.26105
	Equal variances not assumed			4.392	9.800	.001	1.5000	.34157	.73684	2.26316
pH 6.5	Equal variances assumed	6.250	.031	-8.000	10	.000	-2.6667	.33333	-3.40938	-1.92395
	Equal variances not assumed			-8.000	5.000	.000	-2.6667	.33333	-3.52353	-1.80981
pH 7	Equal variances assumed	.625	.448	5.839	10	.000	2.5000	.42817	1.54597	3.45403
	Equal variances not assumed			5.839	8.288	.000	2.5000	.42817	1.51856	3.48144
pH 7.5	Equal variances assumed	.250	.628	1.000	10	.341	.3333	.33333	-.40938	1.07605
	Equal variances not assumed			1.000	9.615	.342	.3333	.33333	-.41343	1.08009

Group Statistics

	Concn.	N	Mean	Std. Deviation	Std. Error Mean
pH 4.5	1.00	6	5.0000	.00000	.00000
	3.00	6	4.6667	.51640	.21082
pH 5	1.00	6	3.0000	.63246	.25820
	3.00	6	2.6667	.51640	.21082
pH 5.5	1.00	6	3.5000	.54772	.22361
	3.00	6	3.0000	.63246	.25820
pH 6	1.00	6	4.5000	.54772	.22361
	3.00	6	2.0000	.63246	.25820
pH 6.5	1.00	6	.0000	.00000	.00000
	3.00	6	2.0000	.63246	.25820
pH 7	1.00	6	4.5000	.54772	.22361
	3.00	6	2.3333	.81650	.33333
pH 7.5	1.00	6	3.0000	.63246	.25820
	3.00	6	3.3333	.81650	.33333

Independent Samples Test

		Levene's Test for Equality of Variances		t-test for Equality of Means						
									95% Confidence Interval of the Difference	
		F	Sig.	t	df	Sig. (2-tailed)	Mean Difference	Std. Error Difference	Lower	Upper
pH 4.5	Equal variances assumed	40.000	.000	1.581	10	.145	.3333	.21082	-.13640	.80307
	Equal variances not assumed			1.581	5.000	.175	.3333	.21082	-.20859	.87526
pH 5	Equal variances assumed	.250	.628	1.000	10	.341	.3333	.33333	-.40938	1.07605
	Equal variances not assumed			1.000	9.615	.342	.3333	.33333	-.41343	1.08009
pH 5.5	Equal variances assumed	.625	.448	1.464	10	.174	.5000	.34157	-.26105	1.26105
	Equal variances not assumed			1.464	9.800	.175	.5000	.34157	-.26316	1.26316
pH 6	Equal variances assumed	.625	.448	7.319	10	.000	2.5000	.34157	1.73895	3.26105
	Equal variances not assumed			7.319	9.800	.000	2.5000	.34157	1.73684	3.26316
pH 6.5	Equal variances assumed	2.500	.145	-7.746	10	.000	-2.0000	.25820	-2.57530	-1.42470
	Equal variances not assumed			-7.746	5.000	.001	-2.0000	.25820	-2.66372	-1.33628
pH 7	Equal variances assumed	1.250	.290	5.398	10	.000	2.1667	.40139	1.27232	3.06101
	Equal variances not assumed			5.398	8.742	.000	2.1667	.40139	1.25457	3.07876
pH 7.5	Equal variances assumed	1.667	.226	-.791	10	.448	-.3333	.42164	-1.27280	.60613
	Equal variances not assumed			-.791	9.412	.449	-.3333	.42164	-1.28082	.61415

Group Statistics

	Concn.	N	Mean	Std. Deviation	Std. Error Mean
pH 4.5	1.00	6	5.0000	.00000	.00000
	4.00	6	1.6667	1.03280	.42164
pH 5	1.00	6	3.0000	.63246	.25820
	4.00	6	.6667	.51640	.21082
pH 5.5	1.00	6	3.5000	.54772	.22361
	4.00	6	3.0000	.63246	.25820
pH 6	1.00	6	4.5000	.54772	.22361
	4.00	6	2.0000	.63246	.25820
pH 6.5	1.00	6	.0000	.00000	.00000
	4.00	6	2.0000	.63246	.25820
pH 7	1.00	6	4.5000	.54772	.22361
	4.00	6	2.3333	.51640	.21082
pH 7.5	1.00	6	3.0000	.63246	.25820
	4.00	6	1.6667	.51640	.21082

Independent Samples Test

		Levene's Test for Equality of Variances		t-test for Equality of Means						
									95% Confidence Interval of the Difference	
		F	Sig.	t	df	Sig. (2-tailed)	Mean Difference	Std. Error Difference	Lower	Upper
pH 4.5	Equal variances assumed	10.652	.009	7.906	10	.000	3.3333	.42164	2.39387	4.27280
	Equal variances not assumed			7.906	5.000	.001	3.3333	.42164	2.24948	4.41719
pH 5	Equal variances assumed	.250	.628	7.000	10	.000	2.3333	.33333	1.59062	3.07605
	Equal variances not assumed			7.000	9.615	.000	2.3333	.33333	1.58657	3.08009
pH 5.5	Equal variances assumed	.625	.448	1.464	10	.174	.5000	.34157	-.26105	1.26105
	Equal variances not assumed			1.464	9.800	.175	.5000	.34157	-.26316	1.26316
pH 6	Equal variances assumed	.625	.448	7.319	10	.000	2.5000	.34157	1.73895	3.26105
	Equal variances not assumed			7.319	9.800	.000	2.5000	.34157	1.73684	3.26316
pH 6.5	Equal variances assumed	2.500	.145	-7.746	10	.000	-2.0000	.25820	-2.57530	-1.42470
	Equal variances not assumed			-7.746	5.000	.001	-2.0000	.25820	-2.66372	-1.33628
pH 7	Equal variances assumed	.625	.448	7.050	10	.000	2.1667	.30732	1.48192	2.85141
	Equal variances not assumed			7.050	9.966	.000	2.1667	.30732	1.48160	2.85174
pH 7.5	Equal variances assumed	.250	.628	4.000	10	.003	1.3333	.33333	.59062	2.07605
	Equal variances not assumed			4.000	9.615	.003	1.3333	.33333	.58657	2.08009

Group Statistics

	Concn.	N	Mean	Std. Deviation	Std. Error Mean
pH 4.5	1.00	6	5.0000	.00000[a]	.00000
	5.00	6	3.0000	.00000[a]	.00000
pH 5	1.00	6	3.0000	.63246	.25820
	5.00	6	2.0000	.63246	.25820
pH 5.5	1.00	6	3.5000	.54772	.22361
	5.00	6	2.5000	.54772	.22361
pH 6	1.00	6	4.5000	.54772	.22361
	5.00	6	2.0000	.63246	.25820
pH 6.5	1.00	6	.0000	.00000	.00000
	5.00	6	3.5000	.54772	.22361
pH 7	1.00	6	4.5000	.54772	.22361
	5.00	6	3.0000	.00000	.00000
pH 7.5	1.00	6	3.0000	.63246	.25820
	5.00	6	.5000	.54772	.22361

a. t cannot be computed because the standard deviations of both groups are 0.

Independent Samples Test

		Levene's Test for Equality of Variances		t-test for Equality of Means						
									95% Confidence Interval of the Difference	
		F	Sig.	t	df	Sig. (2-tailed)	Mean Difference	Std. Error Difference	Lower	Upper
pH 5	Equal variances assumed	.000	1.000	2.739	10	.021	1.0000	.36515	.18640	1.81360
	Equal variances not assumed			2.739	10.000	.021	1.0000	.36515	.18640	1.81360
pH 5.5	Equal variances assumed			3.162	10	.010	1.0000	.31623	.29540	1.70460
	Equal variances not assumed			3.162	10.000	.010	1.0000	.31623	.29540	1.70460
pH 6	Equal variances assumed	.625	.448	7.319	10	.000	2.5000	.34157	1.73895	3.26105
	Equal variances not assumed			7.319	9.800	.000	2.5000	.34157	1.73684	3.26316
pH 6.5	Equal variances assumed			-15.652	10	.000	-3.5000	.22361	-3.99823	-3.00177
	Equal variances not assumed			-15.652	5.000	.000	-3.5000	.22361	-4.07480	-2.92520
pH 7	Equal variances assumed			6.708	10	.000	1.5000	.22361	1.00177	1.99823
	Equal variances not assumed			6.708	5.000	.001	1.5000	.22361	.92520	2.07480
pH 7.5	Equal variances assumed	.625	.448	7.319	10	.000	2.5000	.34157	1.73895	3.26105
	Equal variances not assumed			7.319	9.800	.000	2.5000	.34157	1.73684	3.26316

Group Statistics

	Concn.	N	Mean	Std. Deviation	Std. Error Mean
pH 4.5	1.00	6	5.0000	.00000	.00000
	6.00	6	2.0000	.63246	.25820
pH 5	1.00	6	3.0000	.63246	.25820
	6.00	6	1.5000	.54772	.22361
pH 5.5	1.00	6	3.5000	.54772	.22361
	6.00	6	2.5000	.54772	.22361
pH 6	1.00	6	4.5000	.54772	.22361
	6.00	6	3.0000	.89443	.36515
pH 6.5	1.00	6	.0000	.00000	.00000
	6.00	6	3.0000	.89443	.36515
pH 7	1.00	6	4.5000	.54772	.22361
	6.00	6	.5000	.54772	.22361
pH 7.5	1.00	6	3.0000	.63246	.25820
	6.00	6	1.5000	.54772	.22361

Independent Samples Test

		Levene's Test for Equality of Variances		t-test for Equality of Means						
									95% Confidence Interval of the Difference	
		F	Sig.	t	df	Sig. (2-tailed)	Mean Difference	Std. Error Difference	Lower	Upper
pH 4.5	Equal variances assumed	2.500	.145	11.619	10	.000	3.0000	.25820	2.42470	3.57530
	Equal variances not assumed			11.619	5.000	.000	3.0000	.25820	2.33628	3.66372
pH 5	Equal variances assumed	.625	.448	4.392	10	.001	1.5000	.34157	.73895	2.26105
	Equal variances not assumed			4.392	9.800	.001	1.5000	.34157	.73684	2.26316
pH 5.5	Equal variances assumed			3.162	10	.010	1.0000	.31623	.29540	1.70460
	Equal variances not assumed			3.162	10.000	.010	1.0000	.31623	.29540	1.70460
pH 6	Equal variances assumed	.625	.448	3.503	10	.006	1.5000	.42817	.54597	2.45403
	Equal variances not assumed			3.503	8.288	.008	1.5000	.42817	.51856	2.48144
pH 6.5	Equal variances assumed	10.000	.010	-8.216	10	.000	-3.0000	.36515	-3.81360	-2.18640
	Equal variances not assumed			-8.216	5.000	.000	-3.0000	.36515	-3.93864	-2.06136
pH 7	Equal variances assumed			12.649	10	.000	4.0000	.31623	3.29540	4.70460
	Equal variances not assumed			12.649	10.000	.000	4.0000	.31623	3.29540	4.70460
pH 7.5	Equal variances assumed	.625	.448	4.392	10	.001	1.5000	.34157	.73895	2.26105
	Equal variances not assumed			4.392	9.800	.001	1.5000	.34157	.73684	2.26316