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SELECTION, EFFICACY, ECOLOGICAL CHARACTERIZATION AND FORMULATION OF FUNGAL CONTROL AGENTS AGAINST WATER HYACINTH [*EICHHORNIA CRASSIPES* (MARTIUS) SOLMS-LAUBACH] IN MALI

SÉLECTION, EFFICACITÉ, CARACTÉRISATION ÉCOLOGIQUE ET FORMULATION DES PATHOGENES FONGIQUES CONTRE LA JACINTHE D'EAU DOUCE [*EICHHORNIA CRASSIPES* (MARTIUS) SOLMS-LAUBACH] AU MALI

A dissertation submitted by
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for obtaining the degree of Doctor of
Philosophy (Ph.D.) in Engineering Sciences

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ABBREVIATIONS

IER	Institut d'Economie Rurale
DGCD	Direction Générale de la Coopération au Développement
US$	Dollars of United State of America
CSIRO	Commonwealth Scientific and Industrial Research Organisation
Gx-ABT	Gembloux Agro Bio Tech
BTC	Belgian Technical Cooperation
a_w	Water activity
mg	Milligramme
MS	Mean square
df	Degree of freedom
l	Liter
mm	Millimeter
RH	Relative humidity
DS	Disease severity,
LNE	Laboraoire Nationale des Eaux du Mali
NCBI	National Center for Biotechnology Information
ITS	Internal Transcribed Spacer
USA	United State of America
rRNA	Ribosomal RiboNucleic Acid
DNA	DesoxyriboNucleic Acid
P	Probability
bps	Base pairs
PDA	Potato Dextrose Agar
MA2	Malt Agar 20%
µl	Microliter
BCCM	Belgian Coordinated collections of Microorganisms
MUCL	Mycothèque de l'Université Catholique de Louvain
PhD	Philosophy Doctor
GPS	Global Positionning System
EF1a	Elongation Factor 1 alpha gene
cmdA	Calmodulin gene
act	Actin gene

ABSTRACT

Dagno K., 2011. *Selection, efficacy, ecological characterization and formulation of fungal control agents against water hyacinth [Eichhornia crassipes (Martius) Solms-Laubach] in Mali.* Ph.D. thesis, University of Liege - Gembloux Agro – Bio Tech. 107 p., 16 tabl., 12 fig.

ABSTRACT

Rice and irrigated orchards are considered as the most important food and economical sources in Mali, which watered from Niger River. However, stable productions of rice and orchards products have been limited by many disease, insects, and weeds. Recently, water hyacinth infestation in river of Niger has increased drastically and as affected by the decrease of water flow due to clogging dams and irrigation work. Biological control of weeds is an alternative approach to chemical herbicide use, focusing on the use of native fungal to control or reduce the population of water hyacinth. From 2006 to 2007, 1000 samples consisting of infected parts of water hyacinth were collected from the River Niger (District of Bamako, Segou and Niono with GPS coordinates "12° 40' N, 7° 59' W" ; "13° 26' N, 6° 15' W" and "14° 15' N, 5° 59' W" respectively) in Mali. Fungal belonging to seven genera (116 isolates in all) were recovered from surface-sterilized water hyacinth parts with pronounced blight symptoms. On the basis of *in vivo* pathogenicity tests in which the diseased leaf area percentage was estimated visually and disease severity was estimated (by calculating a disease severity index), three isolates emerged as causing severe disease: *Fusarium* sp. isolate Mln799 (DS = 70%), *Cadophora* sp. isolate Mln715 (70%), and *Alternaria* sp. isolate Mlb684 (71%), identified respectively as *Gibberella sacchari* (Anamorph. *Fusarium sacchari*), *Cadophora malorum*, and *Alternaria* sp. This is the first report to highlight *C. malorum* as a candidate biocontrol agent for water hyacinth. Influence of ecological parameter, water activity (0.880 - 0.960a_w modified par glycerol) and temperature (15-35°C) on viable conidia and mycelial growth rate of *Alternaria* sp. (isolate Mlb684), *F. sacchari* (isolate Mln799) and *C. malorum* (isolate Mln715) were evaluated. Germination and mycelial growth dropped as a_w of the medium decreased. Results showed a significant effect of a_w, temperature and their interaction on mycelial growth ($P < 0.0001$). Growth models for *F. sacchari* (isolate Mln799), *C. malorum* (isolate Mln715) and *Alternaria* sp. (isolate Mlb684) differed slightly, with R^2 values of 93.40, 85.89 and 77.31% respectively. Mycelial growth models, no different between predicted and experimental values was observed. Results confirm the previous finding that a_w has greater influence on growth of *F. sacchari* (isolate Mln799), *C. malorum* (isolate Mln715) and *Alternaria* sp. (isolate Mlb684) than temperature. Analyse of 5 target regions of isolate Mlb684 genome (18 and 28S ribosomal RNA genes, Elongation factor-1 alpha gene, calmodulin gene and actin gene) allowed identifying this isolate as new specie in *Alternaria* genus. The strain has been deposited under accession number MUCL 53159 in the Industrial Fungal & Yeast Collection (BCCM^{TM}/MUCL, Belgium) and DNA sequences have been deposited in GenBank. We have named it "*Alternaria jacinthicola* Dagno & M.H. Jijakli". It was the first sequenced of calmodulin gene reported in *Alternaria* genus. Water hyacinth, paddy and wheat substrates for mass production of these biocontrol agents were evaluated. Greater sporulation of *C. malorum* (isolate Mln715) was observed on water hyacinth substrate (4.08 x 10^7 spores ml^{-1}) following by wheat (1.06 x 10^7 spores ml^{-1}). Biocontrol efficacy of fungal isolates found to be enhanced with unrefined *Carapa procera* (L) oil and refined palm oil supplemented with soybean lecithin in greenhouse. Applying *C.*

malorum (isolate Mln715) and *A. jacinthicola* (MUCL 53159 strain) in vegetable oil emulsion [*Carapa procera* (L) and palm oils] caused 87.02 to 93.13% of damage severity on water hyacinth 6 weeks after treatment. Field trials showed lower efficacy control than greenhouse tests. In host specificity tests, neither *C. malorum* isolate Mln715 nor *Alternaria* sp. isolate Mlb684 showed any pathogenicity towards any member of a panel of 17 crop plants of economic importance in Mali.

Keywords: Bioherbicide, water hyacinth, biocontrol agents, *Alternaria jacinthicola*, *Cadophora malorum*

RESUME

Dagno K., 2011. *Sélection, efficacité, caractérisation écologique et formulation des agents de lutte fongique contre la jacinthe d'eau [Eichhornia crassipes (Martius) Solms-Laubach] au Mali.* Doc. de thèse, Université de Liège - Gembloux Agro - Bio Tech. 107 p., 16 tabl., 12 fig.

RESUMÉ

Au Mali où l'agriculture occupe 90% de la population active, le riz et les productions maraîchères et fruitières en système irrigué constituent les plus importantes sources d'alimentation et de revenus économiques. L'eau de ces systèmes d'irrigation provient du fleuve Niger. Cependant, la sécurité de ces systèmes de culture est fondatalement menacée par la presence de la jcinthe d'eau dans le lit des cours d'eau du fleuve Niger. Ces 20 dernières années l'infestation de cet adventice s'est considérablement accrue au point le débit des barrages d'irrigation. La lutte biologique au moyen des pathogènes fongiques est une alternative approche à l'utilisation des herbicides pour maintriser à long terme la population de la jacinthe d'eau. A cet effet, de 2006 à 2007, une collecte de 1000 échantillons de tissus infectés de cet adventice a été réalisée dans les cours du fleuve Niger envahis par la jacinthe d'eau dans le District de Bamako, Segou et Niono avec respectivement "12° 40' N, 7° 59' O" ; "13° 26' N, 6° 15' O" et "14° 15' N, 5° 59' O" comme coordonées GPS. A partir des échantillons, 7 genres de champignons composés de 116 isolats ont été identifiés. Un test de pathogénicité a été effectué en serre aucours duquel 3 isolats : Mln799 (Fusarium sp.), Mln715 (*Cadophora* sp.) et Mlb684 (*Alternaria* sp.) ont causé respectivement 70, 70 et 71% de sévérité de maladie foliaire. Ces isolats ont été identifiés comme étant *Gibberella sacchari* (Anamorph. *Fusarium sacchari*) (isolat Mln799), *Cadophora malorum* (isolat Mln715), et *Alternaria* sp. (isolat Mlb684) par la Mycothèque de L'université catholique de Louvain-la-Neuve. L'influence des paramètres écologiques : a_w (0.880 - 0.960 a_w modifié par le glycérol) et la température (15-35°C) sur la germination et la croissance de ces 3 pathogènes ont été évalués. La viabilité des spores et le taux de croissance des champignons ont baissé en fonction de la diminution de l'aw du milieu de culture. Les résultats ont montré un effet significatif de aw, la température et leur interaction sur la croissance mycélienne ($P < 0,0001$). Les modèles de croissance pour *F. sacchari* (isolat Mln799), *C. malorum* (isolat Mln715) et *Alternaria* sp. (isolat Mlb684) diffère légèrement, avec des valeurs R^2 de 93,40, 85,89 et 77,31% respectivement. Cependant, pour le modèle de croissance, il n'y a pas eu de différence entre les valeurs prédites et celles expérimentales. Les résultats ont demontré que l'a_w a eu une plus grande influence sur la croissance de *F. sacchari* (isolat Mln799), *C. malorum* (isolat Mln715) et *Alternaria* sp. (isolat Mlb684) que la température. Le séquençage et l'analyse de 5 régions (18 et 28S gènes ribosomiques de l'ARN, gène du Facteur d'élongation alpha-1, les gènes de la calmoduline et de l'actine) du génome de l'isolat Mlb684 ont permis d'identifier ce champignon comme une nouvelle espèce au sein du genre *Alternaria*. C'est le premier séquençage du gène calmoduline dans le genre *Alternaria*. Un spécimen de la culture à été deposé sous le numéro MUCL53159 à la Mycothèque de l'université catholique de Louvain-La-Neuve (Belgique) et les séquences d'ADN on été déposées à GenBank. Cette nouvelle espèce a été appelée "*Alternaria jacinthicola* Dagno & MH Jijakli. En production de masse sur les substrats végétaux, on a obtenu un plus grand rendement en spore de *C. malorum* (isolat Mln715) sur milieu à base de jacinthe ($4,08 \times 10^7$ spores ml^{-1}) suivi par celui du blé ($1,06 \times 10^7$ spores ml^{-1}). L'application *C. malorum* et *A.*

jacinthicola (MUCL53159 strain) dans une formulation d'huiles végétales [*Carapa procera* (L) et l'huile de palme] a causé 87,02 à 93,13% de maladie foliaire sur la jacinthe d'eau 6 semaines après pulvérisation en serre. Les 2 agents de lutte biologique ont été moins efficaces en conditions d'application en plein champ. *Cadophora malorum*, et *Alternaria jacinthicola* n'ont pas montré de pathogenicité sur 15 cultures irriguées économiquement importantes au Mali. Ils n'ont pas été pathogènes non plus sur la pomme et la poire en condition de conservation.

Mots clés : Bioherbicide, jacinthe d'eau, agents de lutte biologique, *Alternaria jacinthicola*, *Cadophora malorum*

GENERAL INTRODUCTION

INTRODUCTION

Water resources in Mali are extremely important for the environment, industry, domestic and agricultural purposes. Rainfall in Mali is highly erratic and variable. So while much of the country is arid, other parts are subjected to frequent flooding. In an attempt to alleviate this situation, numerous dams, weirs and inter-basin transfer schemes have been constructed to ensure sufficient water resources for domestic, agricultural and industrial use. Further, aquatic resources in Mali have become increasingly polluted through urban runoff, incorrect agricultural practices, informal development and the products of industrial wastes (Dagno, 2006).

The alteration of flow regimes and the enrichment of water bodies in Mali have made them susceptible to biological invasion. Indeed, the invasion of rivers, dams and lakes throughout Africa by introduced aquatic vegetation represents one of the largest threats to the socio-economic development of the continent. At present there are five aquatic weeds that are especially problematic in Africa: *Azolla filiculoides* (Lam.) (Red water fern), *Myriophyllum aquaticum* (Vell.) Verdc. (Parrot's feather), *Salvinia molesta* (Mitchell) (Salvinia) and *Eichhornia crassipes* (Mart.) Solms (water hyacinth) (Cilliers et al., 2003). Among them, water hyacinth is the most widespread and damaging aquatic plant species in Africa (Hill, 2003). Water hyacinth also interferes with hydropower generation and obstructs water flow in irrigation channels; besides, facilitating rampant mosquito breeding in the aquatic systems, and fostering (Praveena et al., 2004). This plant was introduced to Africa from South America in the late 1800s (Jones, 2009).

Herbicides (Paraquat, Diquat, Glyphosate, Amitrole, 2,4-D acid) have been largely used to control water hyacinth infestations in water body (Land Protection, 2001). However, their residual toxicity and their deleterious effects required alternative strategies to manage this aquatic weed (Gupta et al., 2002).

In recent years, therefore, the focus shifted to natural enemies of water hyacinth including plant pathogens. In particular, the success of the host-specific *Cercospora rodmanii* in controlling water hyacinth greatly stimulated interest in the management of the weed using fungal pathogens (Freeman, 1984). Recently, a new mycoherbicide named Hyakill has reported on water hyacinth control by de Jong et al. (2003). Despite successful biological control of *Eichhornia crassipes* in greenhouse conditions in USA, Netherland, India and Egypt, no commercial mycoherbicide is available for water hyacinth. The plant is still regarded as problematic in many irrigation regions of Mali.

The specific objectives of this research were (1) to isolate, screen and rank fungi infecting water hyacinth plants on the basis of their pathogenicity and ability to cause damage, and to test the potential of highly pathogenic species as biocontrol agents ; (2). to evaluate the influence of environmental parameters [water activity (a_w), and temperature] on the radial growth rate of the three promissing biocontrol agents ; (3) to evaluate vegetable substrates as mass production support of biocontrol agents and evaluation of effects of vegetable oil emulsion formulation on biocontrol efficacy of the two most adapted bioherbicide agents.

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CHAPTER I

REVIEW OF THE LITERATURE

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Abstract

Water hyacinth (*Eichhornia crassipes*), probably originating from South America, grows between 35th North and South parallels of the planet. It was introduced into several regions of the world as ornamental plant, where it became later one of the most dangerous world water weed. Its infestation can be controlled by physical and chemical treatments. However, these methods are expensive and dangerous for human health and environment. Moreover, following Western legislations example, those of developing countries are highly restrictive against authorised chemical molecules. Consequently, a growing interest was given to alternative solutions, such as biological control. That control using insects and fishes against water hyacinth could be strengthened with the application of mycoherbicides. These mycoherbicides are more respectful for environment and public health and seem to constitute an additional realistic alternative for water hyacinth durable management. Trends in implementation of bioherbicides use in the control of water hyacinth depends primarily on several strategies, most prominently is looking for alternatives to chemical control methods. This method aimed at minimizing hazards resulting from herbicide residue on both human and animal health, and on the ecosystem in general. In addition, one of the major strategies of the biological control concept is attempting to incorporate the biological weed control methods as a component of integrated weed management to achieve satisfactory control results and meanwhile, reduce herbicide application to the minimum extent possible. Several fungal pathogens with mycoherbicide potential (Hyakill™ contains *Sclerotinia sclerotiorum* and *Cercospora rodmanii*, named ABG-5003) have been discovered on disease seed water hyacinth plant, but any have become commercially available in the market. Biological, technological, and commercial constraints have hindered progress. Many of these constraints are being addressed, but there is a critical need to better understanding the biochemical and physiological aspects of pathogenesis of potential bioherbicides. Oil emulsions are recognized as a way to increase both efficiency of application and efficacy of biocontrol agents.

Keywords. Water hyacinth, biocontrol, formulation, mycoherbicide, patent.

I. Introduction

Water hyacinth (*Eichhornia crassipes*) from *Pontederiaceae* family is one of the world's most important aquatic weeds. This noxious weed spreads by vegetative reproduction (Gopal, 1984). Its uncontrollable growth rate and ability to infest a wide range of freshwater habitats have created enormous environmental, agricultural and public healthy problems (Honmura et al., 1998; Charudattan, 1996). Water hyacinth infestations can reduce the availability of water for irrigation, aquaculture, potable water, navigation and recreation, as well as obstruct drainage canal worldwide.

In North America, introduction of water hyacinth in USA is prohibited (U.S.EPA, 1988). Several million of US dollars are spent annually to control hyacinth infestation in USA. From 1980 to 1991, Florida spent more than $ 43 million US. Since that period, water hyacinth control strategies cost annually $ 3 million US and 500,000 US in California (Mullin et al., 2000).

It is difficult to quantify the economic impact due to water hyacinth infestation in developing countries. It has spread throughout Africa causing widespread problems to millions of users of water bodies and water resources. This is especially very severe in Mali and Egypt, where human activities and livelihoods are closely linked to the water systems. Dagno et al. (2007) reported that mechanical management of the weed costs $ 80,000–100,000 US per year in Mali. For Fayad et al. (2001), there were 487 km^2 of irrigation canals and 151 km^2 of lakes which were covered by hyacinth plants in many areas of Egypt. This infestation caused a loss of 3.5×10^{12} m^3 of water per year, corresponding to a sufficient quantity to irrigate approximately 432 km^2 per year.

Among the different methods of water hyacinth control, conventional methods which rely mainly on mechanical/manual removal and use of chemical herbicides, which have generally been found to be inadequate and expensive measures to apply on a large scale. Herbicides have the added disadvantage that they might have adverse environmental effects, and must be applied carefully and selectively (Bateman, 2001). Biological control appears as an efficient alternatives method of water hyacinth sustainable management. The use of bioherbicides to control weeds has gained major prominence (Senthilkumar, 2007). Bioherbicides mainly utilize endemic pathogens that are destructive to the weed and are usually applied in massive doses at vulnerable stages of host growth (El-Morsy et al., 2006). There is however, current interest in the use of plant pathogenic microbes for biological control of weeds. Inert solid carriers, alginate granules, invert emulsions and oil-in-water emulsions have all been considered as vessels for mycoherbicides as they reduce or eliminate the dew requirement for fungal colonization (Hanspeter et al., 1998; Greaves et al., 1998).

Survey of fungal micro-organisms on water hyacinth infesting rivers and other water bodies permitted us to identify many pathogens as potential mycoherbicides. Some fungal pathogens can cause significant reductions in water hyacinth biomass (50-100%) when used as inundating bioherbicide agents (El-Morsy, 2004; Charudattan, 1986; Shabana et al., 1995a). Several good reasons can be considered to use fungal pathogens as biological control agents. This review paper relates biological control approaches in sustainable management systems and highlights challenges, to develop mycoherbicides against water hyacinth.

II. Problems of the water hyacinth, *Eichhornia crassipes*, in the tropical and subtropical areas of the world and control strategies

II.1. Aquatic weed problems in Mali

Mali is a sahelian country is landlocked in West Africa. Its economy relies mainly on the agro-forestry-pastoral activities. Indeed, the agricultural sector employs nearly 75% of the workforce. A Rice and orchard activities contributes to 5% of gross domestic product of Mali and is a key of food security (Dagno, 2006).

Water resources are extremely important for these agricultural components. Mali's rainfall is highly erratic and variable. So while much of the country is arid, other parts are subjected to frequent flooding. In an attempt to alleviate this situation, numerous dams, weirs and inter-basin transfer schemes have been constructed on River Niger to ensure sufficient water resources for agricultural use and production of electricity.

The alteration of flow regimes and the enrichment of River Niger have made them susceptible to biological invasion. The invasion of water bodies, dams and lakes in Mali by introduced aquatic weed represents one of the largest threats to the socioeconomic development of this country (Dembele, 1994). At present there are three aquatic weeds that are especially problematic in Mali (Dembele, 1994). *Pistia stratiotes* L. (water lettuce) is from South America are present in rice irrigation area of Niger Office. *Salvinia molesta* (Mitchell) (salvinia) is another South American native free-floating aquatic fern species which is invasive throughout the world and has been present in Mali since the 1990s (Dembele, 1994). Among them, the most widespread and damaging aquatic plant species is *Eichhornia crassipes* (Mart.) Solms (water hyacinth). The cause of the introduction in Mali of this weed is still unknown.

From 2003 to 2008, the electricity dam has been a loss of 686 hours of electricity production by turbine stop for manual manages of water hyacinth. This control strategies of the weed have cost 1 539 and 19 345 US$ per year to Energy authority and Niger Office respectively.

Moreover, annual income about 100 million US$ of orchard products would be threatened by water hyacinth presence in water bodies in irrigation areas. Cost in public health (malaria and diarrheic disease) would have increased to 2% because water hyacinth may promote the development of vectors for these diseases. Indeed, the management of aquatic plants cost 10 million US$ per year in West Africa.

II.2. Taxonomy, morphology and ecology of water hyacinth

II.2.1. Taxonomy

Kingdom	– *Plantae*	Plants
Subkingdom	- *Tracheobionta*	Vascular plants
Superdivision	- *Spermatophyta*	Seed plants
Division	- *Magnoliophyta*	Flowering plants
Class	- Liliopsida	Monocotyledons

Subclass - *Commelinidae*
Superorder - *Commelinanae*
Order - *Pontederiales* [*Philydrales*]
Family - *Pontederiaceae*
Genus - *Eichhornia*
Species - *Eichhornia crassipes* Water hyacinth

The family *Pontederiaceae* contains 5 genera and 21 accepted taxa overall (Gopal, 1987), of which the following genera are recognized, namely,

Genus *Eichhornia* Kunth – Water hyacinth
Genus *Heteranthera* Ruiz & Payon – Mudplantain
Genus *Monochornia* K.Presl – Monochornia
Genus *Pontederia* L. – Pickerelweed
Genus *Reussia* Endl. – Reussia

and the genus *Eichhornia* contains 6 accepted species (Gopal, 1987), namely:

Species *azurea* Kunth – Anchored water hyacinth
Species *crassipes* (Mart.) Solms – Common water hyacinth
Species *diversifolia* (Vahl) Urban – Variable water hyacinth
Species *paniculata* (Spreng) Solms – Brazilian water hyacinth
Species *paradoxa* Solms – Brazilian water hyacinth
Species *natans* (Beauv.) Solms – African water hyacinth

The distribution of the above four species is as follows (Gopal, 1987), namely: *E. azura*, first reported from Jamaica, is widely distributed with its range overlapping (almost completely) that of *E. crassipes*. This specie is widely distributed in South America. *E. diversifolia*, is largely distributed in Brazil and extends to Surinam, Cuba, Haiti, Puerto Rico and Santo Domingo and *E. paniculata*, occurs mainly in northern Argentina and Brazil. There are two other species of *Eichhornia*, namely *E. natans* (Beauv.) Solms, which is endemic to Africa (Senegal, Sudan, Nigeria and Mali) and *E. paradoxa* Solms which is endemic to Brazil and probably in Caracas (Venezuela) (Gopal, 1987).

II.2.2. Morphology

Water hyacinth is a perennial, aquatic plant, free-floating or anchored in shallow water. Usually 100 – 200mm high, it can extend to 1 meter when growing in dense mats. Roots of floating plants are long and feathery. Leaves of water hyacinth are shiny dark green in colour, in rosettes with distinctive erect swollen bladder-like petioles (Figure1). Flowers are pale violet or blue, in flowered spikes with each flower measuring about 50mm in diameter. The upper petal has a prominent dark blue, yellow-centred patch. Fruit consists of capsules with very fine seed (Henderson, 2001). The root structure of the plant gives it a feathery appearance, due to it being adventitious and fibrous. As much as 50% of a single plant's biomass could be made up of the root structure (Gopal, 1987).

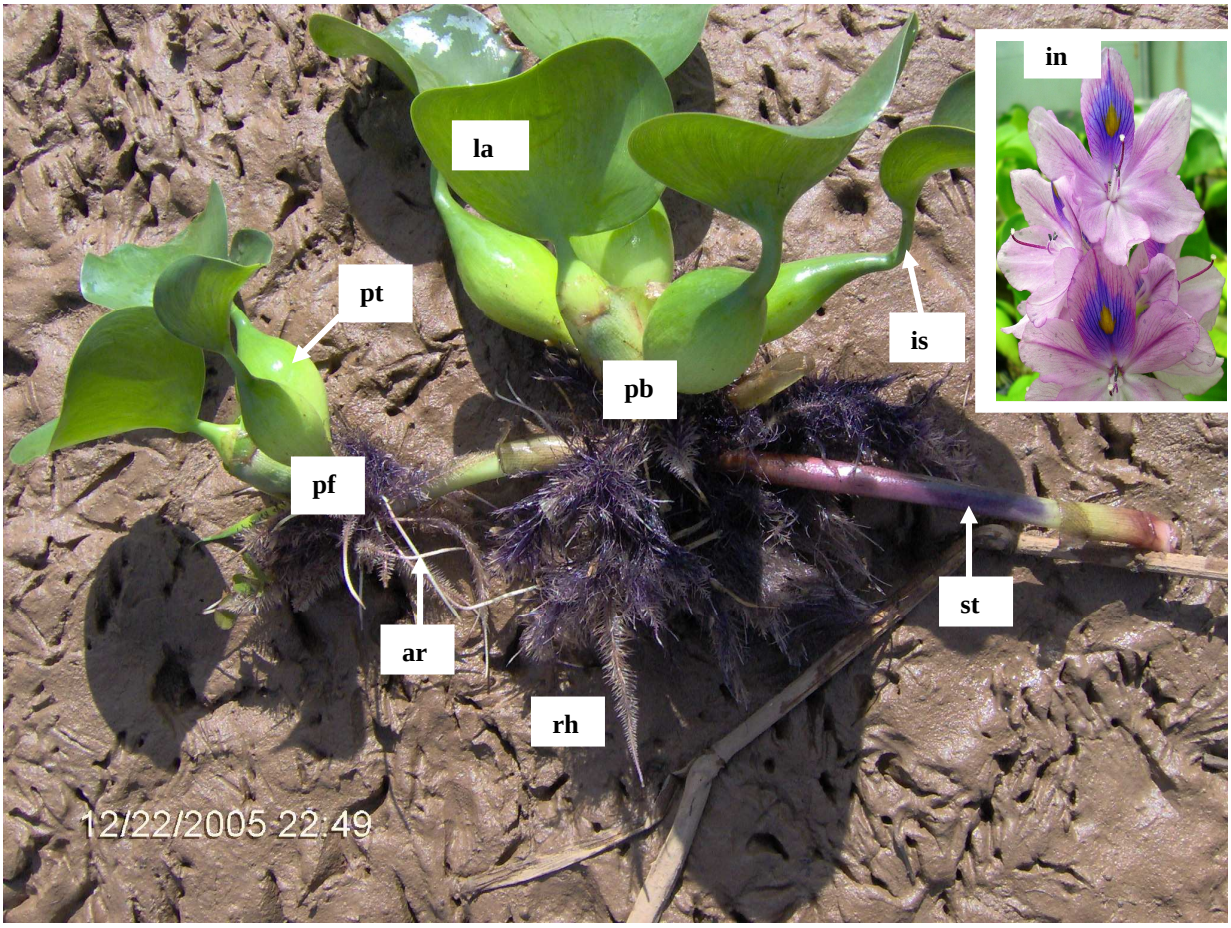

Figure 1. Morphology of the water hyacinth. pf = plant daugther; pb = petiole with bulb, rivet washer forms produced in a floating state; ar = adventitious root; in = fl orescence; is = stalk of the leave; la = blade of leave; pt = petiole of the leave; rh = rhizome; st = stolon (Dagno, 2006).

II.2.3. Ecology

Water hyacinth occurs in both highly acidic and alkaline waters but more luxuriant growth is observed in near neutral water bodies. The water may be clean and poor in major nutrients as in most rivers and reservoirs, or may be highly polluted with large amounts of nutrients and organic matter as in severage lagoons (Gopal, 1987) and many aquatic systems in Mali. One hectare of water hyacinth plants under optimum conditions could absorb the average daily nitrogen and phosphorus waste production of over 800 people (Jones, 2009). Invasive species are widely accepted as one of the leading causes of biodiversity loss and can have significant effects on resource availability and can suppress or enhance the relative abundance of native species, without necessarily being the driving force behind community change (Didham et al., 2005). A dense cover drastically reduces and may prevent light penetration of water. Without light, phytoplankton and submerged plants can not photosynthesize. Oxygen levels decrease and carbon dioxide increases, with catastrophic effects on the aquatic fauna (Howard et al., 1998). Populations of fish can be reduced or eliminated, as well as other animals (Gratwicke et al., 2001). Few invaded ecosystems are free from habitat loss and disturbance, leading to uncertainty as to whether dominant invasive

species are driving community change or are passengers along for the environmental ride (MacDougall et al., 2005).

Water hyacinth has a direct impact on aquatic systems water flow by slowing it by 40 to 95% in irrigation channels (Jones, 2009), which may cause severe flooding. This could have a detrimental effect on the ecology of the system. In addition it is suggested that mats of water hyacinth lower temperatures, pH, bicarbonate alkalinity and dissolved oxygen content and increase the free carbon dioxide content, and nutrient levels (Jones, 2009).
Dense mats of water hyacinth reduce light to submerged plants and thereby reduce oxygen levels in aquatic communities (Gopal, 1984). Invertebrate community composition is altered (McVea et al., 1975). During periods of strong winds, mats of water hyacinth drift and scour indigenous vegetation. This in turn destroys both plant and wildlife habitats (Gowanloch et al., 1948). Exotic species (water hyacinth) that invade systems represent a threat to that ecosystem and could directly modify an ecosystem, causing a cascading effect for resident biota e.g. space (Crooks, 2002).

II.2.4. Habitat

Water hyacinth can be located worldwide in a variety of different habitats. These include habitats varying from shallow ponds, possibly temporary, to large lakes and even fairly fast flowing rivers (Gopal, 1987). Where the plant is situated in shallow water bodies it does not have to contend with excessive wave action and varying depths of water. The velocity of water also plays a significant role in the plant's habitat. Climatic conditions vary within a system and will have an affect on the ecology of the plant itself. Water hyacinth can be located in both natural water and artificially made aquatic systems. However, it does not occur in aquatic systems with an average salinity greater than 15% of sea water (Junes, 2009).

Water hyacinth is able to remove high levels of nutrients from water, which then influences the plant's growth form eg. plant established in or near to a sewage outlet, compared to those plant's established in a low nutrient habitat. The plant grows prolifically in nutrient enriched waters and new plant populations form from rooted parent plants. Wind and current assists to distribute them. Excessively large mats can be formed. The root system, as well as the above water structures of the plant, forms a habitat for organisms. However, large mats of water hyacinth are capable of negatively affecting the original habitat.

The feathery roots of water hyacinth provide a suitable habitat and substrate for specific periphytic microphytes. In addition, a large variety of invertebrates are particularly associated with the roots hanging in the water as they provide a habitat, and may possibly trap them from the water column. In some cases a few organisms have been reported to be specifically associated with water hyacinth mats. Water hyacinth mats also provide a suitable habitat for germination and establishment of the seedlings of a number of emergent species (Gopal, 1987).

II.3. Growth and reproduction

Water hyacinth, growing in ideal situations, has an incredible mechanism to outgrow any native species occurring in the system. It is capable of reproducing vegetatively, which is its primary reproductive method. This is carried out from the 'mother' plant via stolons. During periods of high wind and wave action, plants are able to disperse and colonize other areas of the system. Penfound et al. (1948), further record that 10 adult plants are capable of producing some 655 360 daughter plants in a single growing season. Gopal (1987) reports that water hyacinth has the capacity to increase sevenfold in 50 days, that the edge of mat extends by 60 cm per month, that 2 plants can multiply to 1 200 plants in 120 days, that the surface area increases by an average of 8% per day and that the surface mat can double every 6.2 days.

Another method used by water hyacinth to reproduce, is sexually. Sexual reproduction is reported to be limited (Gopal, 1987). Plants are capable of flowering throughout the year, should environmental factors be suitable. Penfound et al. (1948), reported that a water hyacinth ovary may produce up to 500 ovules, but rarely sets more than 50 seeds per capsule in trials that they performed. Barrett (1980), performed trials and concluded that 44.2 seeds per capsule were the average, with a high seed germination rate of 87.5% on average. Penfound et al. (1948), further recorded that upwards of 900 capsules have been counted in an area of one hectare. This equates to 45 million seeds per hectare. Of interest is the fact that they further suggest that pollination by insects rarely occurs, but that self-pollination is a common phenomenon during the wilting stage. Water hyacinth seed can lay dormant for many years, until the correct climatic conditions arise, when it may then germinate. Seed can remain dormant for up to 20 years (Gopal, 1987). Very few seeds germinate on the mat, as they are lost in the detritus build-up, or sink due to being heavier than water.

II.4. Origin of water hyacinth and distribution

In 1823, the German naturalist C. von Martius discovered the species, while carrying out floral surveys in Brazil. He named it *Pontederia crassipes*. Solms included it in the *Eichhornia* genus, 60 years later, as had previously been described by Kuntz in 1829. However, a collector by the name of von Humbolt had already collected specimens from Colombia in 1801, together with the species *azurea* (Gopal, 1987). The reason for the world-wide distribution of this weed varies, but generally it has coincided with the plant's ornamental properties or as feed (Ding et al., 2001).

The native range of *E. crassipes* (Gopal, 1987) in South America includes Argentina, Brazil, Paraguay, Uruguay, Bolivia, Ecuador, Colombia, Chile, Guyana, Surinam and Venezuela. It has spread to Panama, Nicaragua, Honduras and El Salvador in Central America. There appears to be several schools of thought when it comes to the actual point of origin / dispersal of *E. crassipes*.

In North America, it is believed to have been introduced in 1884 at the Cotton States Exposition in New Orleans, Louisiana. It subsequently spread across the south-eastern United States to Florida in 1895 and California in 1904 (Center et al., 2005). Water hyacinth is most prolific in the south-eastern states, as well as being recorded in Hawaii (Center et al., 2005). Water hyacinth has, since its introduction to Louisiana, spread to Alabama, Arkansas, Arizona, California, Colorado, Florida, Georgia, Hawaii, Kentucky, Louisiana, Missouri,

Mississippi, North Carolina, New York, Oregon, South Carolina, Tennesse, Texas, Virginia and Washington, (USDA–NRCS, www.USDA.gov) in other words to about 50% of the State in the U.S.A.

It was introduced into Asia towards the end of the 19th Century via Japan and Indonesia (Ueki et al., 1975) where it naturalized in rice fields in Indonesia (Backer, 1951), where it was grown as an ornamental plant in the Botanical Gardens. In India it first appeared in Bengal at the beginning of 1890. It has also established in Taiwan and China, as early as 1901 as a good fodder plant (Ding et al., 2001). It was first noticed in Australia in Brisbane, Sydney and Grafton in the 1890's and has since spread to all mainland States and Territories. As in other countries, here to it is believed to have been introduced as an ornamental plant. In Australia it was introduced as an aquarium plant. It has in addition spread to Papua New Guinea, where it was first recorded from dredge ponds in old gold fields of Bulolo in 1962 (Harley et al., 1996*)*.

Holm et al. (1977*)* record the fact that both New Zealand and Bangladesh also have water hyacinth infestations. Burton (2005) records that many islands in the Pacific Ocean also have weed infestations. Europe has also been affected by water hyacinth, where it was introduced as an ornamental plant in Portugal. The first record of it was made in 1939. First documented records for Spain are for 1989 (Têllez et al., 2008). Gates (2000) and Harper (2000) reported that water hyacinth has also been observed in the wild in Britain.

Water hyacinth was originally introduced outside of its home range, due to the lack of understanding of the plant invasive properties and the immense ecological negative impacts that it would have on fresh water ecosystems. In more modern times, lack of enforcement of relevant legislation or in some cases the lack of any relevant legislation has assisted in the degradation of freshwater systems. The lack of general public awareness has also been a factor.

The invasion of rivers, dams and lakes throughout Africa by introduced aquatic vegetation represents one of the largest threats to the socioeconomic development of the continent (Cilliers et al., 2003). Africa has been particularly affected by the introduction and spread of water hyacinth (Table 1). Climatic suitability modelling suggests that, with the exception of the drier areas of the continent (Sahara and Kalahari deserts), water hyacinth would be able to infest most of the continent and the fact that it does not occur in all countries in Africa is more due to it not having being recorded or not having spread there, rather than it not being able to establish (Wise et al., 2007).

The first recorded introduction of water hyacinth onto the continent was for Egypt in the period 1889 – 1892, during the reign of Khevede Tawfiq (Gopal, 1987). It is believed to have been introduced as an ornamental plant. Water hyacinth occurs throughout the Nile Delta and is believed to spreading southwards, due to the construction of the Aswan Dam, which has slowed the river down (Gopal 1987).

The second record for the continent is for South Africa in 1908 (Stent, 1913). Water hyacinth is believed to have been introduced as an ornamental aquatic plant for garden ponds and aquaria, owing to its attractive flowers (Ashton et al., 1979). In the case of water hyacinth, a warning of what was likely to happen, was printed as early as 1913 (Jacot, 1979).

Thirdly, Zimbabwe recorded water hyacinth infestations in 1937. The first record was from the Mukuvisi River in Harare and the plant only attained its pest status in the early

1950's on Lake Chivero (Chikwenhere et al., 1999). In the period 1941 to 1960, a further ten African countries (Table 1) recorded water hyacinth infestations, namely: Angola (1942), Benin (1942), Burundi (1957), Congo (1950-1951), the Democratic Republic of Congo (1952), Ethiopia (1956), Mozambique (1942), Rwanda (1957), Sudan (1954) and Tanzania (1955).

There appears to be a slump in water hyacinth introductions, in the period from 1961 to 1980, and this is due to the fact that a further eight countries are recorded to have water hyacinth infestations but no accurate data are available to ascertain when the first recordings were made. It is, however, acknowledged that some of these infestations could have taken place in the period 1981 to 2000. However, a further four African countries, published their first records of water hyacinth infestations during 1961 - 1980, namely: Central African Republic (1970), Malawi (1960's), Senegal (1963) and Zambia (1965). Nine African countries recorded water hyacinth infestations in the period 1981 to 2000, namely: Burkina Faso (1989), Cote d' Ivoire (1980's), Ghana (1984), Kenya (1982), Niger republic (1987), Nigeria (1982), Togo (1987) and Uganda (1988).

Both intentional introductions (ornamental) and unintentional introductions (rivers flowing from one country to another) of water hyacinth have occurred throughout Africa, since the first intentional introduction into Egypt.

Table 1. Distribution of water hyacinth in African countries.

Country	First Recorded	Reference
Angola	1942	Gopal, 1987
Benin	1942	Gopal, 1987.
Burkina Faso	1989	Ouedraogo et al., 1999
Burundi	1957	Navarro et al., 2000
Cameroon	?	Barrett, 1989
Central African Republic	1970	Gopal, 1987
Congo	1950 – 51	Gopal, 1987
Côte d' Ivoire	1980's	Koffi et al., 1999
Democratic Republic of Congo	1952	Greathead et al., 1993
Egypt	1879-1892	Fayad, 1999
Equitorial Guinea	?	Barrett, 1989
Ethiopia	1956	Navarro et al., 2000
Gabon	?	Barrett, 1989
Ghana	1984	Greathead et al.,1993
Guinea-Bissau	?	Barrett, 1989
Kenya	1982	Ochiel et al., 1999
Liberia	?	Barrett, 1989
Malawi	1960's	Navarro et al., 2000
Mali	1990's	Dembele, 1994
Mozambique	1942	Gopal, 1987.
Niger Republic	1987	Ouedrago et al., 1999.
Nigeria	1982	Charudattan et al., 1995.
Rwanda	1957	Navarro et al., 2000.
Senegal	1963	Gopal, 1987.
Sierra-Leone	?	Barrett, 1989.
South Africa	1908	Stent, 1913.
Sudan	1954	Navarro et al., 2000.
Tanzania	1955	Mallya, 1999.
Gambia	?	Barrett, 1989.
Togo	1987	Ouedrago et al., 1999.
Uganda	1988	Ogwang et al., 1999.
Zambia	1965	Mailu et al., 1999.
Zimbabwe	1937	Chikwenhere et al., 1999

II.5. Control of water hyacinth

The best form of weed control is prevention. Always treat weed infestations when small, do not allow weeds to establish. Weed control is not cheap but it is cheaper now than next year, or the year after. Proper planning ensures you get value for each dollar spent. The best approach is usually to combine different methods. Control may include; chemical, mechanical, fire and biological methods; combined with land management changes. The control methods you choose should suit the specific weed and your particular situation.

II.5.1. Mechanical control

Physical removal is most effective for small infestations and any such attempt should be made before flowering and seed set in October. Plant may survive for a short time on moist river or creek banks. After removing the plant, follow up with drying and burning to destroy reproductive organs. This will prevent introduction following rainfall and subsequent flooding.

II.5.2. Biological control

Table 2 contained insect species have been introduced from South America and released by CSIRO since 1975. The two weevil species are *Neochetina eicchomiae* and *Neochetina bruchi* and the two moth species are *Niphograpta albiguttalis* (previously known as *Sameodes albiguttalis*) and *Xubida infusella*. The weevil *N. eicchomiae* has been the most successful so far, and has played a key role in removing large infestations in tropical areas of the state. The adult is black, 5 mm long and feeds on the leaves making small scars. Eggs are laid in the bulbous leaf stalks and the larvae tunnel through the plant tissues. The damaged tissues are then attacked by bacteria and fungi. The plant becomes waterlogged and under heavy attack and will die. The life cycle of the weevil takes three months and the insect is inactive over winter. The other weevil, *N. bruchii*, is active through the winter and so complements *N. eicchomiae*. This weevil was first released in south-east Queensland in 1990 and in north Queensland in 1991. Field testing of this weevil is still ongoing and it appears that *N.bruchii* is effective. It appears that introducing both of them is the best possible option, because their life cycles complement each other. However, both weevils are much less effective in sub-tropical and cooler areas. Biological control is most effective on large areas of water hyacinth. However, it may take up to 10 years to achieve satisfactory control by this means.

Moreover, several fungal species were isolated on water hyacinth in world wide (Figure 2). Among theme *Cercospora rodmanii*, *Alternaria alternata* and *A. eichhorniae* are recognized as potential mycoherbicide agents for this weed (Shabana, 2005; Babu et al., 2003 ; Charudattan, 1996).

There are herbivorous fish that are also used for control water hyacinth proliferation. We are Chinese carp, *Ctenopharyngo idella*, as well *Tilapia melanopleura* and *T. mossambica* carps feed on hyacinth. Chinese carp is used in biological program management in USA. However, this specie is not specific to water hyacinth plant (Gopal, 1987).

Table 2. Insects succesfully introduced and used in the biological control against the water hyacinth in the world (Dagno, 2006).

Ordres	Espèces	Région d'établissement
Coléoptère	*Neochetina bruchi*	Floride, Louisine, Californie, Argentine, Australie, Inde, Soudan, Bénin, Nigéria, Afrique du Sud, Malawi, Ouganda.
	Neochetina eichhorniae	Texas, Argentine, Australie, Inde, Soudan, Bénin, Zimbabwe, Ghana, Kenya, Tanzanie, Ouganda, Malawi, Afrique du Sud.
Lépidoptère	*Niphograpta albiguttalis* (syn. *Sameodes albiguttalis*)	Floride, Louisiane, Mississipi, Bénin, Panama, Soudan, Australie
	Bellura densa (syn. *Arzama densa*)	Floride, Louisiane, Argentine
Acarien	*Orthogalumna terebrantis*	Egypte, Zambie, Fiji, Zimbabwe

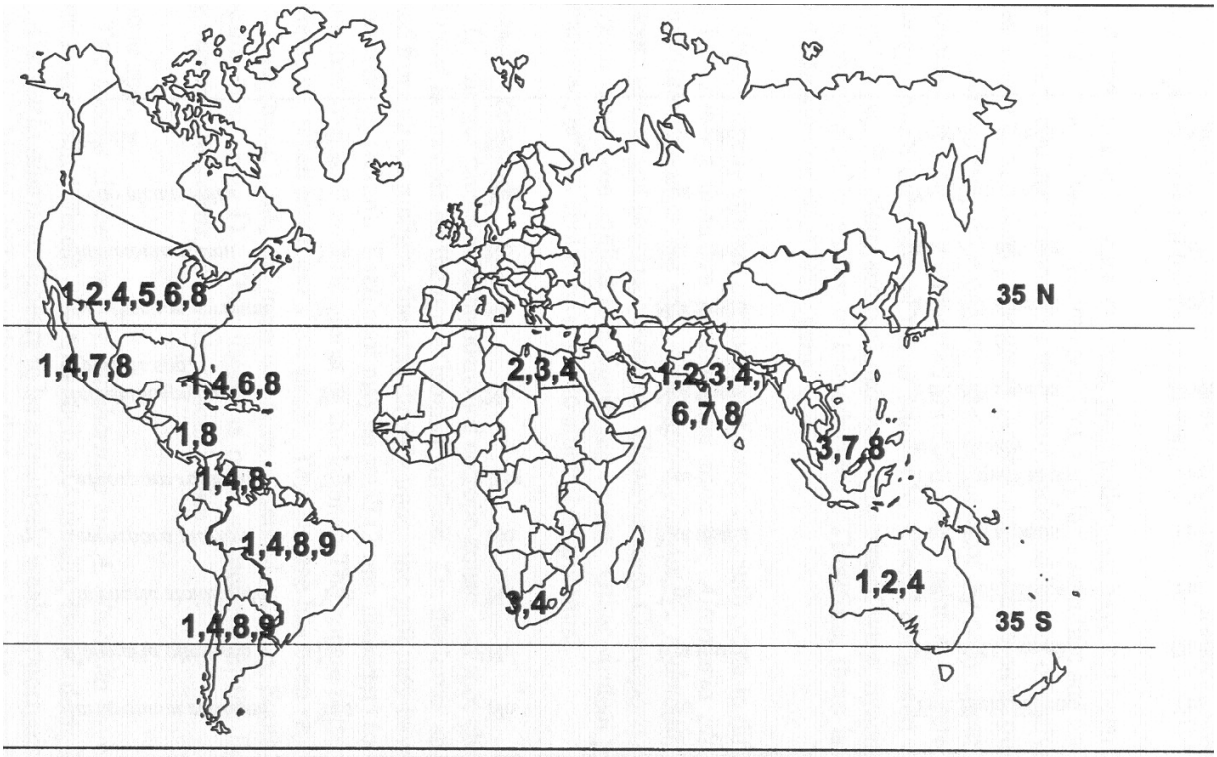

Figure 2. Distribution of main pathogens on *Eichhornia crassipes* throughout the world. 1 : *Acremonium zonatum* ; 2 : *Alternaria alternata* ; 3 : *Alternaria eichhornia* ; 4 : *Cercospora piaropi* ; 5 : *Cercospora rodmanii* ; 6 : *Helminthosporium/Bipolaris* spp. ; 7 : *Myrothecium roridum* ; 8 : *Rhizoctonia solani* ; 9 : *Uredo eichhorniae*. (Charudattan, 1996).

II.5.3. Herbicide control

Before using any herbicide always read the label carefully. All herbicides must be applied strictly in accordance with the directions on the label. When treating water that is used for irrigation purposes, the withholding period should be followed in accordance with the

label recommendations. Spraying an entire heavy infestation can cause water hyacinth to sink and result in pollution from the rotting weed. Large masses of the rotting weed will use all the oxygen in the water leading to fish and wildlife kills. This problem can be avoided by spraying strips of the weed or by mechanically removing much of the weed before spraying. For this reason it is vital to destroy scattered plants when they appear rather than delaying treatment until the entire water body has been choked.

Heavy infestations on shallow water bodies should be physically removed to avoid putrefaction of the water. Diquat (Regione) is the only product registered for use in water storage areas used for human consumption (Land Protection, 2001). Note that 14 days must elapse after treatment before water can be consumed.

II.5.4. Integrated control

Integrated control is a sensible strategy that includes the combination of mechanical, biological and chemical methods. The different methods complement each other. First make certain that the weevils are established on the infestation, and then carry out a spray program using a selective herbicide. Spraying sections of the infestation at a time will concentrate the insects on the remaining weed, they will destroy the remainder. Mechanical removal of dead plants will avoid water quality degradation by masses of rotting weed.

II.5.5. Control by transformation

Table 3 show different products obtained by transformation of water hyacinth plants. These product used in large scale may reduced the weed mats on water bodies.

Table 3. Various techniques of water hyacinth transformations as means of control (Dagno, 2006).

Valorisation	Countries
Wastewater treatment	China, USA, Sri Lanka, Argentina, Brazil, Egypt, India, France
Paper	Bangladesh, Uganda
Board construction	Inde
Fishing nets and ropes	Bangladesh
Basketry	India, Fiji, Philippines, Uganda
Charcoal briquet	Kenya
Livestock feed, compost	Bangladesh, Birmania, Mali

II.6. Conclusion

Originally introduced to Africa in particularly in Mali as an aquatic ornamental plant, water hyacinth has become a major pest of rivers and dams. Not only does it destroy native habitats, but it also seriously depletes water bodies of oxygen, increases water loss and provides a breeding ground for mosquitoes. An integrated control program that includes prevention and control (by physical, biological and chemical means) is the most effective management strategy. Ideally, dead plants are removed to avoid putrefaction of the water body.

III. Actual statuts of mycoherbicide development against aquatic weed, *Eichhornia crassipes* (Martius) Solms-Laubach: Succes and challenge

III.1. Biocontrol strategy of weeds

Biocontrol strategy of weed is delivery method based on the use of natural enemies to suppress the growth of a weed or to reduce a weed population (Cruttwell, 2000). There are two basic strategies to implement the biological control of weeds by pathogens. The introduction of foreign pathogenic organisms, often called the classical approach, and an augmentative bioherbicides strategies, where the pathogenic organisms are already present (native or introduced) and their population is increased by mass rearing (El-Sayed, 2005). In epidemiological terms, these approaches are also often described as inoculative and inundative strategy respectively (Hasan et al., 1990).

The inoculative or classical approach implies the control of invasive weeds by introducing a few populations of control organisms from the weed's natural habitat. These pathogens are released only on a small part of the total infested area and the control is achieved by gradual spread of the initial population (El-Sayed, 2005).

Classical method present some disadvantages. Among them, they are the high initial costs, number of natural enemies for each target weed species is limited and dissemination of biocontrol agent (BCA) cannot be controlled after release in nature. At those, a successful control strongly depends on favourable conditions promoting an effective increase in the population of BCA, and the establishment of epiphytotics to reduce the target weed population (El-Sayed, 2005).

Inundative or bioherbicide strategies use periodic releases of an overabundant supply of the controlling organism to suppress the entire weed population. Such pathogens or biological agents are generally "manufactured", formulated, standardised, packed and registered like chemical herbicides (Auld et al., 1995). One group of such biological agents with promising potential for weed control is mycoherbicides. The use of inundative approach may give several advantages in control of weeds. Indeed, the plant pathogen is mass-produced and applied in high dose to the target weed in much the same way as a chemical herbicide. Finally, the large number of biological control agents is intended to immediately suppress the target population.

This review was directed towards the development of a mycoherbicide instead of the inoculative control because, to introduce fungus pathogen which does not exist in a new area after the weed has already settled is dangerous compared to a local indigenous fungus multiplied and slackened in the region (Charudattan, 2005).

III.2. Bioherbicides registered until 2010

There is a long history of research on microbial control agents, it is not always appreciated that obtaining an active isolate is only the beginning of a series of activities necessary for implementing the use of a new mycoherbicide (O'Connell et al., 1996). There are important issues to consider including: mass production, delivery systems and 'laboratory to field' studies, strategies for use, registration and commercialisation (Bateman, 2001).

The level of research reports in bioherbicides research has increased tremendously since the early eighties of the last century. Both the number of weeds targeted for control and candidate pathogens studied has increased. Practical registered or unregistered uses of bioherbicides have also increased worldwide. Likewise, the numbers of U.S. patents issued for bioherbicidal use of fungi and their technology have been increased, perhaps foretelling an increased reliance on bioherbicides in the

future (El-Sayed, 2005). Table 4 illustrated registered bioherbicides and their current worldwide status.

Table 4: Submit of a patent application in biocontrol of weeds.

Countries and registered date	Products and pathogens	Target weeds	Current statut
USA, 1960	*Acremonium diospyri*	Persimmon (*Diospyros virginiana*) trees in rangelands	Status unknown
China, 1963	Lubao, *Colletotrichum gloeosporioides* f. sp. *cuscutae*	Dodder (*Cuscata* spp.) in soybeans	Probably still available
USA, 1981	DeVine®, *Phytophthora palmivora*	Strangler vine (*Morrenia odorata*) in citrus orchards	Status unknown, may no longer be marketed
USA, 1982	Collego™, *Colletotrichum gloeosporioides* f. sp. *aeschynomene*	Northern joint vetch (*Aeschynomene virginica*) in rice & soybeans	Distributed since 2003, but rice producers are showing renewed interest
USA, 1983	CASST™, *Alternaria cassiae*	Sickle pod & coffee senna (*Cassia* spp.) in soybeans & peanuts	No longer available due to lack of commercial backing
USA, 1987	Dr. BioSedge, *Puccinia canaliculata*	Yellow nutsedge (*Cyperus esculentus*) in soybeans, sugarcane, maize, potato & cotton	Product failed due to uneconomic production system & resistance in some weed biotypes, no longer available
Canada, 1992	BioMal®, *Colletotrichum gloeosporioides* f. sp. *malvae*	Round-leaved mallow (*Malva pusilla*) in wheat, lentils & flax	No longer commercially available but made on request
Canada, 2004	Myco-Tech™ paste, *Chondrostereum purpureum*	Deciduous tree pecies in rights of way & forests	Commercially available
USA, 2005	Smolder, *Alternaria destruens*	Dodder species: in agriculture, dry bogs & ornamental nurseries	Only just registered. Company planning to do more field trials & then market it in 2007
Canada, 2007	Sarritor, *Sclerotinia minor*	Dandelion (*Taraxacum officinale*) in lawns/turf	Commercially available

III.3. Factors influencing mycoherbicide efficacy

The efficacy of a mycoherbicide depends on the establishment of the disease during the primary infection and also on the complete control of the weed via the secondary infection (Yang et al., 1993).

III.3.1. Primary infection

The importance of environmental conditions such as dew and temperature, on primary infection was demonstrated by Walker et al. (1983) and Auld (1993). The environmental conditions vary from field to field and year to year. Differences in primary infection among years and locations were observed during experiments with *Colletotrichum gloesporioides* (Penz) f.sp. *malvae* (BioMal, Philom Bios) and *Alternaria alternata* used against water hyacinth and round leaf mallow in India respectively (Babu et al., 2003). Even for Collego, which is used in free moisture to fields, low levels of initial infections have been recorded in some years.

However, the low initial infections due to varying effect of environmental parameters may be offset by high capacity of dispersal and secondary infections of these pathogens for the control efficacy.

III.3.2. Secondary infection

Numerous mycoherbicide studies indicate the importance of secondary infection and subsequent dispersal for effective control, both requiring time. Boyette et al. (1979) reported that anthracnose disease caused by *C. gloeosporioides* f.sp. *jussiaea*, which has an incubation period of 3-5 days, required 28 days to progress disease from 29% (primary infections) to 94% on winged water primrose plant in rice field. In the same field experiment, fungal dispersal was evident, because 25% of the plants in untreated plots were infected, even at a distance of 100 m from inoculated plants.

Several authors have so reported the importance of secondary infection to appear highly disease in weed population. According to Hassan et al. (1992), *Stagonospora* sp. required 3 week after spraying to develop severe disease on *Calystegia sepium* (L.). Elwakil et al (1989) reported that *Alternaria alternata*, a potential mycoherbicide for water hyacinth in an aquatic environment, required 2 month to achieve lethal levels, although the incubation period to observe first symptoms was 12 days. In Brazil, *Helminthosporium* sp. required 36 days post-application to defoliate 73% of inoculated wild poinsettia (*Euphorbia heterophyllia* L.) plants in soybean fields. Field studies by Charudattan (1986) and Morris (2004) have clearly demonstrated that the diseases caused by fungal pathogens progressed from 5% to 90% plant mortality 5 week and 2 months respectively after pulverization. The important role of secondary infection on control efficacy was also demonstrated for commercial mycoherbicides. Experiments with mycoherbicide BioMal on *Malva pusilla* L. showed that at high inoculum concentrations, the control increased from 30-50% at 22 days after application to about 90% at crop harvest. Dispersal of inoculum was evident from the severe disease levels in control plots. Another mycoherbicide, Collego required up 5 week to killing *Aeschynomene virginica* L.

III.4. Promising bioherbicides for water hyacinth

Only two mycoherbicides have been developed to control water hyacinth with the intention to be commercialized. The first herbicide has been registered by US-EPA (United State Environmental Protection Agency) under the patent US4097261 in USA (Freeman and Charudattan, 1984). This product contains *Cercospora rodmanii*, it is a fungal specific pathogen for water hyacinth (Te Beest, 1991). The Abbott Laboratories of USA developed an experimental formulation of *C. rodmanii*, named ABG-5003, against this weed (Praveena et al., 2004).

The second mycoherbicide is called Hyakill™ and contains *Sclerotinia sclerotiorum*. It has been submitted to European Patent Office (de Jong et al., 2003). *S. sclerotiorum* is not specific pathogen on water hyacinth; it is recognized as pathogenic fungal on several crop plants (bean, sunflower, carrot and other dicotyledonous plant families). This reason may be her disadvantage at obtaining European Patent Office authorization of wide use.

Several research groups have identified promising microbial agents that might be used as biopesticides against water hyacinth (Table 5). Fungal species contained in table 2 are evaluated for host specificity, biocontrol efficacy and formulation efficiency (Shabana, 1997; Babu et al., 2003; El-Morsy, 2006). Among them, *A. eichhorniae* and *A. alternata* have been extensively studied for biocontrol.

Table 5. Promising bioherbicides for water hyacinth in worldwide.

Weeds target	Potential biocontrol agents	Country of bioherbicide developped
Eichhornia crassipes	*Alternaria. eichhornia*	Egypt
-/-	*A. alternata*	India, Egypt
-/-	*Fusarium pallidoroseum*	India
-/-	*F. chlamydosporum*	Egypt, India
-/-	*Cercospora piaropi*	USA, Mexico
-/-	*Myrothecium roridum*	USA, India, indonesia, Malaisia
-/-	*Uredo eichhorniae*	Egypt, Brazil, Argentina, Uruguay
-/-	*Rhizoctonia solani*	USA, Egypt
-/-	*Acremonium zonatum*	USA, Egypt, Asia, Mexico
-/-	*Dreschlera* sp.	Egypt
-/-	*Phoma* sp.	Egypt

III.4.1. *Alternaria eichhorniae*

Alternaria eichhorniae was reported on water hyacinth in 1984 in Egypt. It appeared to be specific of this aquatic weed (Shabana et al., 2001). The fungal was also after discovered on water hyacinth in Australia, Bangladesh, Indonesia, and South Africa. *A. eichhorniae* is being developed as a mycoherbicide for controlling water hyacinth in Egypt (Shabana, 1995). The symptom of disease are discrete necrotic foliar spots (oblong, 2 - 4 mm long) surrounded by a bright yellow halo.

Shabana et al. (1997) reported that fresher mycelial inoculum (4 weeks old) was more virulent than older inoculum (9 or 16 weeks old). Another report showed biocontrol efficacy of *A. eichhorniae*; inoculum concentrations above 10% of mycelial were all equally effective in controlling waterhyacinth at 100% level (weed kill) (Shabana et al., 2001). This biocontrol agent formulated in invert or oil emulsions showed that all of the invert and oil emulsion induced higher levels of disease on water hyacinth plants than the aqueous formulation under low relative humidity in greenhouse. *A. eichhorniae* formulated in cottonseed oil emulsion caused 100% control of water hyacinth 7–13 weeks after application in field (Shabana, 2005).

However, some authors have been reported that *Spinacea oleracea*, *Cucumis sativus*, *Cucurbita pepo*, *Helianthus annuus*, *Ricizus communis*, *Daucus carota*, *Allium cepa*, *Raphanus sativus*, *Phaseolus vulgaris*, *Ficus carica* and *Lycopersicon esculentum* were shown to be susceptible to *A. eichhorniae* (Babu et al., 2002). This factor of crops susceptibility is unfavourable in large scale use for this mychorbicide.

III.4.2. *Alternaria alternata*

A. alternata is a cosmopolitan fungus and has been isolated from almost all habitats (Guo et al., 2004). The fungus has been described as a pathogen of water hyacinth in Bangladesh (Bardur, 1978), Australia (Galbraith et al., 1984), Egypt (Elwakil et al., 1989; Shabana et al., 1995; EL-Morsy, 2004), and India (Aneja et al., 1989; Babu et al., 2003). This fungal species induces spots and lesions mainly on leaves and less severely on stolons and finally leads to complete death of the plant (Babu et al., 2003).

The formulation of spores in an oil emulsion (10% oil in water) enhanced the efficacy to control water hyacinth plants, the necrotic leaf area of inoculated plants increased as the length of exposure to 100% relative humidity (RH). Severe disease developed on plants inoculated with 10^6 spores ml^{-1} in oil emulsion caused 79% plant tissue death (El-Morsy, 2004). Water hyacinth was susceptible to the fungal at all stages of growth tested; however, older plants were more susceptible than younger ones. The toxin produced by *A. alternata* is known to play an important role in the pathogenesis of the blight disease of water hyacinth (Mohan et al., 2003).

The most and probable limitation of mycoherbicide based on *A. alternata* model is that the fungal is associated with several leaf blight disease on cotton and other economic crops (bean, sunflower, rice, cucumber and peanut) (Bashan et al., 1991).

III.5. Bioherbicide development challenge for water hyacinth

A number of challenges are encountered in the formulation of promising BCAs isolated from water hyacinth. It is necessary to include good market potential, ease of production and application, adequate product stability and shelf life during transportation as well as in storage and guaranteed propagule viability and efficacy over the long term (Boyetchko et al., 1999). Some reasons why BCAs have met with limited commercial success are difficulty of production, sensitivity to UV light and desication, requirements of high humidity for infection, insufficient performance over a wide range of environmental conditions, and lack of appropriate formulation (El-Sayed, 2005).

Formulation is recognized as a way to increase both efficiency of application and efficacy of the biocontrol agent (Evans et al., 2001). Oil emulsion formulations, in particular,

may reduce dew requirements (El-Morsy, 2006; Shabana, 1997) and the number of spores required (Egley et al., 1995). Formulations should be used to improve product stability, bioactivity, and delivery (i. e., ability to mix and spray the product) as well as to integrate the biopesticide into a pest management system (Charudattan, 2001). Other important characteristics of a successful formulation are convenience of use, compatibility with end-user equipment and practices, and effectiveness at rates consistent with agricultural practices (Boyetchko et al., 1999).

For foliar BCAs as *A. eichhorniae* and *A. alternata*, environmental factors that influence plant infection and disease development are temperature, free moisture or dew period, and protection against UV irradiation and desiccation (El-Morsy, 2006; Shabana, 2005). The inclusion of novel synergists in bioherbicide formulations could take them past the point of research, and into the development of efficacious, reliable, and economical products for the marketplace (Bateman, 2001). All of these parameters need to be taken into consideration when developing an appropriate mycoherbicide for the control of water hyacinth as production and formulation of fungal mycroherbicides.

III.6. Conclusion

Several fungal candidates exist for control of water hyacinth infestation, and preliminary research into biological characterizations has been conducted on these fungi for several decades. The literature is replete with reviews on the subject. Despite all of this research and expense poured into development of biocontrol agents, very few have been successful and fewer still have persisted in the marketplace. Several BCAs have been failed, and often for one of multiple common reasons: production problems, lack of adequate shelf life of formulations under warehouse temperatures, lack of an economic viable delivery system, or loss of virulence of the product before reaching the target. Therefore, there is a critical need to better understand the mode of action of bioherbicides involved in host-pathogen interactions which consequently leads to enhance of virulence of pathogen and/or suppress water hyacinth plant's defence.

In fact, there are two major epidemiological components contributing to control efficacy of bioherbicides: a window of temperature and moisture affecting the number of initial infections and the subsequent dispersal and infection of the pathogen within the target weed population. Currently, most research with bioherbicides does not address the importance of secondary infection. This may be because, conceptually, we treat bioherbicides as chemical herbicides. The environmental dependency of BCAs limits control efficacy in variable environments. Consequently, the environmental conditions play a basic role in guiding the mode of action of bioherbicides. In addition, bioherbicides require several complex and often specific interactions between fungal and water hyacinth. This complexity of interactions is one explanation for the unpredictability and inconsistency often associated with bioherbicides restricted commercial bioherbicides to irrigated systems when applied as an aerial spray. Higher constraint for major promising fungal (*A. eichhorniae* and *A. alternata*) as a biocontrol agent for water hyacinth are their dependence on ecological parameters. A number of challenges are encountered in the formulation, including good market potential, ease for production and application, adequate product stability and shelf life during transportation as well as in storage and guaranteed propagule viability and efficacy over the long term. Oil formulations may resolve environment moisture dependence of BCA.

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CHAPTER II

FUNGI OCCURRING ON WATER HYACINTH [*EICHHORNIA CRASSIPES* (MARTIUS) SOLMS-LAUBACH] IN NIGER RIVER IN MALI AND THEIR EVALUATION AS MYCOHERBICIDES

FUNGI OCCURRING ON WATER HYACINTH [*EICHHORNIA CRASSIPES* (MARTIUS) SOLMS-LAUBACH] IN NIGER RIVER IN MALI AND THEIR EVALUATION AS BIOHERBICIDES

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Abstract

One hundred sixteen fungal isolates in seven genera were recovered from water hyacinth plants having pronounced blight symptoms collected in Mali. Isolation frequency of the genera was: *Curvularia* (60.32%), *Fusarium* (42.92%), *Alternaria (*11.6%), *Coniothyrium* (11.6%), *Phoma* (3.48%), *Stemphylium* (3.48%), and *Cadophora* (1.16%). On the basis of *in vivo* pathogenicity tests in which the diseased leaf area percentage and disease severity were visually estimated using a disease severity index, three isolates, *Fusarium* sp. Mln799, *Cadophora* sp. Mln715 and *Alternaria* sp. Mlb684 caused severe disease. These were later identified as *Gibberella sacchari* Summerell & J.F. Leslie, *Cadophora malorum* (Kidd & Beaumont) W. Grams, and *Alternaria* sp. respectively. This is the first report to highlight of *C. malorum* as a candidate biocontrol agent against water hyacinth. Neither *C. malorum* Mln715 nor *Alternaria* sp. Mlb684 in host specificity tests showed any pathogenicity towards 17 crop plants of economic importance in Mali.

Key words: Biocontrol agents, mycoherbicide, native fungi, water hyacinth.

II.1. Introduction

Water hyacinth [*Eichhornia crassipes* (Martius), Solms-Laubach; *Pontederiaceae*] is a free-floating aquatic weed, native to the Amazon Basin in South America (El-Morsy 2004). According Tellez et al. (2008), it is distributed across the tropics and subtropics between 39°N and 39°S (Figure 1). Man has clearly been the main agent responsible for spreading this species around the world, as its entry into Africa, Asia, Australia, and North America coincided with the arrival of the vessels of the first explorers or with historically documented human activities (Shabana 1997).

Water hyacinth is considered the world's worst aquatic weed (Lata and Dubey 2010). As in many tropical and subtropical regions worldwide, water hyacinth creates serious agricultural and navigation problems in District of Bamako in Mali (Figure 1). The plant not only affects irrigation, water flow, water use, and navigation, it also poses a health risk by enabling the breeding of mosquitos, bilharzias, and other human parasites (Adebayo and Uyi 2010). Water quality is affected as well, by the increasing accumulation of detritus in the water (Morsy 2004). Fishing can be affected because of the competitive advantage given to trash fish species in weed-infested waters. In many instances, fish are killed when oxygen levels are depleted through plant respiration and decomposition of senescent vegetation. The water hyacinth problem is particularly severe in the Niger Delta and in the irrigation systems of the "Office du Niger" (Niger Office) (Dembélé 1994). Several billion dollars are spent each year by the "Office du Niger" and "Energie du Mali" to control this weed in the River Niger (Dagno et al. 2007).

Chemical, physical, and biological means are used to control water hyacinth infestations. In Mali, chemical (2-4 D) and mechanical control methods have been used since 1997 to manage this weed (Dembélé and Diarra, 1997); however these methods provide only a temporary management solution. Chemical methods can be dangerous for humans as well as animals, as people drink river water in Mali and use it to prepare food. For this reason, and in a sustainable management perspective, an integrated approach appears necessary.

In recent years, attention has focused on biological control, which could provide a cost-effective, environmentally safe solution to the water hyacinth problem. The biological control of weeds by means of plant pathogens has indeed gained acceptance as a practical, safe, and environmentally beneficial weed management method applicable to agro-ecosystems (Boyette et al., 2007, Charudattan, 2005). Most emphasis has been on fungal pathogens as biocontrol agents (Shabana 2005; Vincent 2001). Several highly virulent fungi are known to cause diseases of water hyacinth; the best known being *Acremonium zonatum* (Sawada) W. Gams, *Alternaria alternata (*Fr.) Keissler, *A. eichhorniae* Nag Raj & Ponnappa, *Bipolaris* spp., *Fusarium chlamydosporum* Wollenw & Reinking, *Helminthosporium* spp., *Cercospora piaropi* Tharp, *Myrothecium roridum* Tode ex fr., *Rhizoctonia solani* Kühn, and *Uredo eichhorniae* Gonz.-Frag. & Cif. (Morsy, 2004; Naseema et al., 2004; Charudattan, 2001). Among these, *A. eichhorniae*, *C. piaropi*, *A. alternata*, and *F. chlamydosporum* have been studied most extensively (Shabana, 2005; Babu et al., 2002).

The aim of the present study was to survey and identify indigenous phytopathogenic fungal isolates of water hyacinth in Mali, with a view to developing them as bioherbicides. We report on the occurrence, pathogenicity, and host specificity of the significant fungal water hyacinth pathogens found in 2006 and 2007 in Mali.

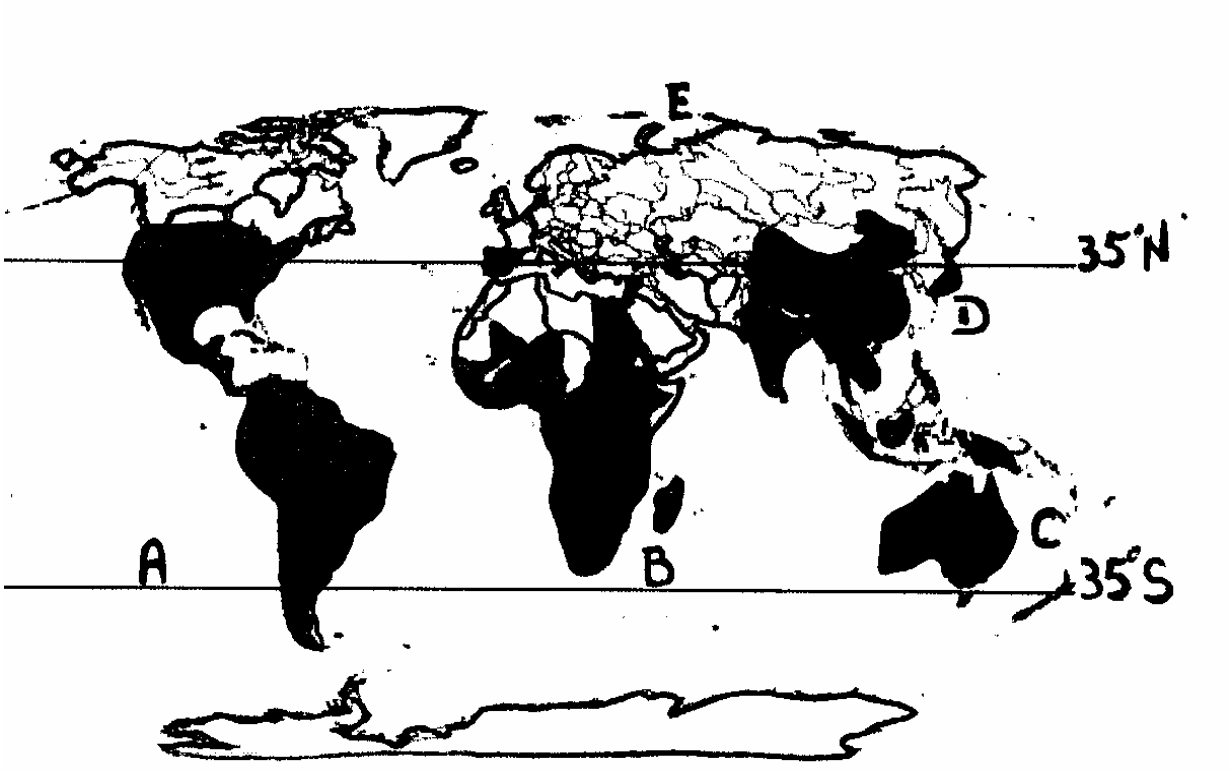


Figure 1. Areas infested by water hyacinth in worldwide. A: American continent, B: African continent, C: Oceania, D: Asia continent, E: Europe continent.

II.2. Material and Methods

II.2.1. Habitat

The study areas were located in the District of Bamako, Segou, and Niono regions with GPS coordinates "12° 40' N, 7° 59' W" ; "13° 26' N, 6° 15' W" and "14° 15' N, 5° 59' W" respectively. The Niono region is the main area for irrigated rice culture and vegetable and fruit production. Bamako, Segou, and Niono have different climates. Bamako (south Mali) has five rainy months with 70% relative humidty (RH), Segou (central Mali) has 4 rainy months with 60% RH, and Niono (exact centre of Mali) has three rainy months with 55% RH (yearly averages). All of these regions are infested with water hyacinth.

II.2.2. Sampling procedure, isolation and fungal classification

From 2006 to 2007, one thousand samples consisting of infected parts of water hyacinth (petiole and leaf) were collected from the River Niger in the District of Bamako (Sites 1, 2, and 3 about 3 km apart), Sebougou lake in Segou (Site 4), and the evacuation canal of wastewater from households (a reservoir for irrigation) of Niono (Site 5). Samples were transported to the laboratory in clean plastic bags and stored at 4° C until examined.

Stored plant parts were scrubbed under running water to remove surface debris, dissected into small segments (approximately 1 × 1 cm), and surface-sterilized by sequential immersion in 96% ethanol for 30 seconds, 14% hypochlorite for 30 seconds and rinsed in sterile water for 1 min. Surface-sterilized segments (4 segments/plate) were plated on Potato Dextrose Agar (PDA, Merck, Darmstadt, Germany) supplemented with 0.5mg streptomycin. Three plates were used for petioles and leaves. The plates were incubated at 25°C for 5 to 7 days. Fungi that developed on the plant pieces were isolated and pure cultures were obtained by the single-spore or hyphal-tip technique, depending on the type of fungal isolate.

Identification of the different fungal genera was based on morphological characteristics of each growing microbial colony, ad described by Lacap et al. (2003) and Ainsworth et al. (1973). Fungi having distinctive characteristics of saprophytes (i.e. rapid growth and sporulation on PDA plates and isolates in the genera *Penicillium, Aspergillus,* and *Trichoderma* were excluded from further consideration after their initial isolation (Martinez and Charudattan 1998). The retained fungal isolates were identified by the Industrial Fungal & Yeast collection (BCCM/MUCL-Louvain-la-Neuve, Belgium).

Based on their frequency of occurrence, the fungal genera isolated were classified as very frequent (> 20%), frequent (10-20%), or infrequent (< 10%), as reported by El-Morsy (2004). The fungal isolates were stored at the Plant Pathology Unit of Gembloux Agro-Bio Tech, University of Liege Wallonia, Belgium.

II.2.3. Environmental factors

Water samples were collected from the water hyacinth infested sites where diseased plants were collected. Water mineral levels and pH were determined at each site along with

temperature and RH. Water samples were analysed by the National Laboratory Water of Mali (LNE). Temperature and RH measurements were performed *in situ* with a thermohygrometer (Thermohygrometer HT5B, W-TECH, 38300 RUY-MONTCEAU, France).

II.2.4. Pathogenecity test

During experiments, the initial conidial inoculum was taken from petri-dish cultures on PDA medium, preserved at 4°C for no more than 6 months, and then subcultured at 25°C on different culture media before use. Healthy water hyacinth plants were collected from natural infestations in the River Niger in Mali and maintained in greenhouse of Gembloux Agro-Bio-Tech, University of Liege.

In a preliminary screen, the fungal pathogens were tested for their effects on 8-cm^2 leaf fragmens cut from healthy, 15- to 20-day-old healthy water hyacinth plants. Each fragment was placed on the surface of a petri plate containing agar supplemented with furfurylaminopurine-6 (1 mg ml^{-1}). Spore suspensions (20 ml) were prepared from the fungal isolates (2 x 10^6 spores ml^{-1} in 5% Tween 20), and manually sprayed manually onto a set of nine leaf fragments. After inoculation, the petri plates were immediately sealed with Parafilm to prevent water loss and incubated at 25°C with a 16-h photoperiod for 4 weeks. After a preliminary screen to detect the most virulent isolates, the pathogenicity of selected isolates was evaluated by inoculating a set of healthy water hyacinth plants maintained in 15 l plastic pots under greenhouse conditions. The nine most virulent isolates were selected for a second screening on whole, 15- to 20-day-old healthy water hyacinth plants. Fourteen day-old cultures of the fungal isolates were used in the test. Three leaves of each plant were inoculated by spraying with a 20 ml spore suspensions (5 x 10^6 spores ml^{-1} in 5% Tween® 20). Inoculated plants were covered with plastic for 72 h and then maintained in incubator room (85 ± 5% RH at 25°C with a 16-h photoperiod). The room RH was maintained by a humidificator (Humidificator DELONGHI UH800E, MANUTAN, 95506 GONESSE, France). The plants were rated for disease symptoms (see scoring scale above) after 6 weeks incubation. The experiment was repeated three times.

Leaf fragments and plants were rated for disease symptoms including leaf spots, leaf lesions, and leaf death after 4 and 6 weeks incubation respectively. The impact of the pathogens was determined by assessing the type of damage (disease severity, DS). DS was determined for each leaf on a scale of 0 to 9, where 0 = healthy, and 9 = 100% diseased as described by Morsy 2004. Values for individual leaves were summed and averaged to derive DS for a whole plant. Finally, isolates were categorized into four groups: “Mild”, < 25% of diseased leaf area; “Low Moderate”, 26-50% of leaf area covered by disease; “Hight Moderate”, 51-75% of diseased leaf area; “Severe”, > 75% of diseased leaf area.

Data were subjected to one-way ANOVA performed with SAS software 9.1 (SAS Institute, Cary, NC, USA). When effects were significant, Duncan’s multiple range tests was employed for mean separation.

II.2.5. Host specificity tests

The host specificity of the selected fungi also was tested in greenhouse. The test included, in addition to three ecotypes of water hyacinth and the invasive aquatic fern *Salvinia molesta* Mitchell, several non-weed plant species chosen for their economic and ecological

importance (Martinez et al. 2001). Non-aquatic plants were grown in pots filled with aseptic commercialized soil free of any fungi. For aquatic plants, the soil contained in 8 l plastic pots was replaced in 15 l plastic pots with water. Each pot contained one plant. The experiment was replicated three times. Inoculation of the water hyacinth ecotypes and the aquatic fern was done by spraying, whereas the fungus was delivered to the crop plants by watering with a suspension containing 5×10^6 spores ml^{-1} simulating irrigation conditions. The plants were monitored for disease symptoms every week for 4 weeks.

II.3. Results and Discussion

II.3.1. Biodiversity

To our knowledge, this is the first extensive survey of pathogens infecting water hyacinth in Mali. Our survey included all sections of the River Niger and all irrigation areas where water hyacinth is reported as a serious problem. Water hyacinth plants in the main irrigation canal were found to be infected by various fungi and displayed a wide variety of symptoms. The symptoms initially appeared as small necrotic spots and developed into a leaf blight spreading over the leaf surface and the petiole. The infecting fungi (116 isolates of filamentous fungi of the phylum *Ascomycetes*) were identified as belonging to 7 known genera (Table 1) on the basis of their morphological characteristics and the arrangement and structure of their conidia.

The genera *Curvularia* (60.32% of the isolates) and *Fusarium* (42.92%) were classified as "very frequent", *Alternaria (*11.6%) and *Coniothyrium* (11.6%) as frequent, and *Phoma* (3.48%), *Stemphylium* (3.48%), and *Cadophora* (1.16%) as infrequent. Sixty-seven isolates were obtained from infected petioles and 49 from leaves. *Curvularia, Fusarium,* and *Stemphylium* appeared widely distributed over all infested areas of Mali, whereas *Alternaria* was found only in Bamako and *Cadophora* and *Phoma* only in Niono.

Previous reports on the mycobiota of water hyacinth indicate that the genera *Fusarium*, *Curvularia*, and *Alternaria* are frequently isolated from this weed (Praveena and Naseema 2004; Evans and Reeder 2001), and that *Alternaria* and *Fusarium* are particularly common (El-Morsy 2004; Praveena and Naseema 2004; Babu et al. 2003).

Table 1**.** List of fungi isolated from different aerial parts of water hyacinth in Mali.

Fungi	Localities						Total count	Frequency of occurence
	Bamako		Segou		Niono			
	Part of plant							
	Petiole	Leaf	Petiole	Leaf	Petiole	Leaf		
Fusarium sp.	11	13		1	6	6	37	42.92
Curvularia sp.	19	7	3	1	11	11	52	60.32
Coniothyrium sp.	5	2	1			2	10	11.60
Alternaria sp.	5	5					10	11.60
Phoma sp.					2	1	3	3.48
Stemphylium sp.	1				2		3	3.48
Cadophora sp.					1		1	1.16
Total	41	27	4	2	22	20	116	

II.3.2. Environmental factors

Temperature, RH, pH and mineral levels recorded for the areas infested with water hyacinth are shown in Table 2. Tellez et al. (2008) reported that the number of daughter plants of this weed is greatest at 25 to 40°C and an RH of 15 to 75%. According to Mandryk and Wein (2006), pH must be between 6 and 8 with the plant showing maximum growth at pH 7. Temperature and pH ranges recorded at the infested sites in Mali (26 to 35°C and pH 6.72 to 7.79) are thus nearly optimal for the growth and vegetative reproduction of water hyacinth.

Factors associated with water pollution (e.g. wastewater from households, industries and agriculture) most likely also contribute to high water hyacinth infestations in Mali.

Table 2**.** Environmental parameters of areas infested by water hyacinth in Mali.

Parameter	Site*				
	S1	S2	S3	S4	S5
Water pH	7.79	7.20	6.72	6.95	7.12
Air relative humidity (RH)	65	60	60	55	55
Air temperature	26	28	27	30	35
Na^+ mgl^{-1}	305	175	105	98	150
Ammoniacal N mgl^{-1}	18.50	35.20	10.40	5.32	41.90
NO_3^- mgl^{-1}	12.80	10.20	13	8.70	11.30

* S1: reserve lake of the hydroelectric dam of Sotuba (Bamako); S2: waste water canal of Badalabougou (Bamako); S3: canal of the Marietou hotel (Bamako); S4: Sebougou lake (Segou) in the central delta of River Niger; S5: wastewater canal in Niono.

II.3.3. Pathogenicity testing of fungal isolates

In the preliminary screen, all 116 fungal isolates were tested *in vitro* on leaf fragments of water hyacinth plants. All the fungal isolates were able to infect the plant and produce some disease symptoms (Table 3). Disease started as small necrotic spots and developed into a leaf blight that tended to spread over the leaf, however, only nine pathogens produced symptoms over more than 50% of the total foliar area by the end of the 4-week incubation period. These were: Mls214 (*Curvularia* sp.), Mln285 (*Curvularia* sp.), Mln286 (*Fusarium* sp.), Mlb603 (*Fusarium* sp.), Mlb633 (*Curvularia* sp.), Mlb682 (*Alternaria* sp.), Mlb684 (*Alternaria* sp.), Mln715 (*Cadophora* sp.), and Mln799 (*Fusarium* sp.).

Disease severity values were calculated over the 4-week period for all 116 isolates (Table 3). The DS values at the end of the experiment for the nine most damaging pathogens were: 50%, 51%, and 50% for *Curvularia* sp. isolates Mls214, Mln285, and Mlb633 respectively, 52%, 50%, and 70% for *Fusarium* sp. isolates Mln286, Mlb603, and Mln799 respectively, 55% and 69% for *Alternaria* sp. isolates Mlb682 and Mlb684 respectively, and 72% for *Cadophora* sp. isolate Mln715.

The second screening was carried out *in vivo* using the nine fungal isolates displaying the highest antagonistic activity *in vitro*. Among them, only isolates Mln799 (*Fusarium* sp.), Mln715 (*Cadophora* sp.), and Mlb684 (*Alternaria* sp.) showed a DS of at least 70% after a 6-week incubation (Figure 2). According to El-Morsy 2004, disease incidence is considered "Hight Moderate" if DS= 51-75% and "severe" if we DS > 75% of leaf area covered by disease. Pictures of the disease on leaves are shown in Figure 3.

The DS was ranged from 40% to 47% for isolate Mls214 (*Curvularia* sp.) Mlb602 (*Fusarium* sp.) and Mlb632 (*Fusarium* sp.). This suggests that they are not strictly pathogenic and that they could be saprotrophs. Several strains of *Curvularia*, *Fusarium*, and *Alternaria* are reported to be biotrophic fungi, living within the host without seriously altering its physiology (Evans and Reeder, 2001).

Alternaria and *Fusarium* are ubiquitous fungal genera and have been isolated from almost all habitats infested with water hyacinth worldwide (Evans and Reeder, 2001; Martinez and Lopez, 2001). These genera include many species or strains that may be

pathogenic towards several crops (Babu et al., 2003; Morsy et al., 2000). They also include some strains that appear specifically pathogenic towards water hyacinth. Among them, *A. eichhorniae*, *A. alternata*, and *F. pallidoroseum* (Cooke) Sacc. have been reported as promising biological control agents against water hyacinth in Egypt and India (Shabana, 1997; Morsy, 2004; Naseema et al., 2004).

This is the first report to mention of the genus *Cadophora* on water hyacinth. This genus, however, is recognized as pathogenic towards corn, rice, apple, and pear (Frisullo, 2002; Benbow et al., 2002).

The present results highlight *Fusarium* sp. isolate Mln799, *Cadophora* sp. isolate Mln715, and *Alternaria* sp. isolate Mlb684 as potential bioherbicides for use in controlling water hyacinth in Mali. These isolates were later identified as *Gibberella sacchari* Summerell & J.F. Leslie, *Cadophora malorum* (Kidd & Beaumont) W. Grams, and *Alternaria* sp. respectively by Industrial Fungal & Yeast collection (BCCM/MUCL - Louvain-la-Neuve, Belgium). Because of its growth at 37°C (human body temperature), isolate Mln799 (*G. sacchari*) was eliminated from our collection and from further planned investigations.

Table 3: Pathogenicity of fungi collected from water hyacinth in Mali (tests applied to detached leaves of water hyacinth)

Isolate number	Genus	Water hyacinth response DS
Mlb436	*Fusarium* sp.	70
Mlb78	*Fusarium* sp.	52
Mlb455	*Fusarium* sp.	50
Mln776	*Fusarium* sp.	49
Mlb11	*Fusarium* sp.	43
Mlb22	*Fusarium* sp.	42
Mlb324	*Fusarium* sp.	42
Mlb498	*Fusarium* sp.	41
Mlb310	*Fusarium* sp.	39
Mln799	*Fusarium* sp.	38
Mln286	*Fusarium* sp.	38
Mlb603	*Fusarium* sp.	36
Mln745	*Fusarium* sp.	36
Mln959	*Fusarium* sp.	33
Mlb127	*Fusarium* sp.	33
Mlb411	*Fusarium* sp.	33
Mlb115	*Fusarium* sp.	32
Mlb697	*Fusarium* sp.	32
Mlb167	*Fusarium* sp.	31
Mlb114	*Fusarium* sp.	31
Mlb340	*Fusarium* sp.	30
Mln906	*Fusarium* sp.	29
Mlb523	*Fusarium* sp.	29
Mlb326	*Fusarium* sp.	28
Mln977	*Fusarium* sp.	27
Mln700	*Fusarium* sp.	27
Mln885	*Fusarium* sp.	26
Mlb130	*Fusarium* sp.	23
Mln236	*Fusarium* sp.	21
Mlb335	*Fusarium* sp.	20
Mlb139	*Fusarium* sp.	20
Mls200	*Fusarium* sp.	18
Mlb639	*Fusarium* sp.	16
Mlb355	*Fusarium* sp.	16
Mln890	*Fusarium* sp.	16
Mlb101	*Fusarium* sp.	15
Mln277	*Fusarium* sp.	12

DS, Disease severity, “Mild”, < 25% of diseased leaf area; “Low Moderate”, 26-50% of leaf area covered by disease; “Hight Moderate”, 51-75% of diseased leaf area; “Severe”, > 75% of diseased leaf area.after 6-week test period.

Table 3 continued 1

Isolate number	Genus	Water hyacinth response DS
Mln285	*Curvularia* sp.	51
Mls214	*Curvularia* sp.	50
Mlb633	*Curvularia* sp.	50
Mlb332	*Curvularia* sp.	45
Mlb109	*Curvularia* sp.	38
Mlb179	*Curvularia* sp.	33
Mlb180	*Curvularia* sp.	33
Mlb55	*Curvularia* sp.	32
Mlb178	*Curvularia* sp.	32
Mlb501	*Curvularia* sp.	31
Mlb344	*Curvularia* sp.	29
Mln279	*Curvularia* sp.	27
Mln960	*Curvularia* sp.	27
Mln963	*Curvularia* sp.	27
Mlb500	*Curvularia* sp.	25
Mls205	*Curvularia* sp.	24
Mlb304	*Curvularia* sp.	24
Mls215	*Curvularia* sp.	23
Mlb301	*Curvularia* sp.	23
Mln917	*Curvularia* sp.	23
Mlb40	*Curvularia* sp.	22
Mlb170	*Curvularia* sp.	22
Mln825	*Curvularia* sp.	22
Mlb171	*Curvularia* sp.	21
Mln830	*Curvularia* sp.	21
Mln835	*Curvularia* sp.	21
Mln898	*Curvularia* sp.	21
Mln970	*Curvularia* sp.	21
Mln709	*Curvularia* sp.	20
Mln838	*Curvularia* sp.	20
Mln893	*Curvularia* sp.	20
Mln896	*Curvularia* sp.	20
Mln926	*Curvularia* sp.	20
Mln892	*Curvularia* sp.	19
Mln913	*Curvularia* sp.	19
Mlb52	*Curvularia* sp.	18
Mln887	*Curvularia* sp.	18
Mlb70	*Curvularia* sp.	16

DS, Disease severity, “Mild”, < 25% of diseased leaf area; “Low Moderate”, 26-50% of leaf area covered by disease; “Hight Moderate”, 51-75% of diseased leaf area; “Severe”, > 75% of diseased leaf area.after 6-week test period.

Table 3 continued 2

Isolate number	Genus	Water hyacinth response DS
Mln762	*Curvularia* sp.	16
Mlb141	*Curvularia* sp.	15
Mlb152	*Curvularia* sp.	15
Mlb10	*Curvularia* sp	14
Mlb66	*Curvularia* sp.	14
Mlb69	*Curvularia* sp.	14
Mln241	*Curvularia* sp.	14
Mlb322	*Curvularia* sp.	14
Mlb24	*Curvularia* sp.	13
Mlb432	*Curvularia* sp.	13
Mln281	*Curvularia* sp.	12
Mlb35	*Curvularia* sp.	11
Mlb526	*Coniothyrium* sp.	47
Mln757	*Coniothyrium* sp.	21
Mlb360	*Coniothyrium* sp.	19
Mln749	*Coniothyrium* sp.	19
Mls210	*Coniothyrium* sp.	18
Mlb440	*Coniothyrium* sp.	18
Mlb486	*Coniothyrium* sp.	16
Mlb187	*Coniothyrium* sp.	15
Mlb627	*Coniothyrium* sp.	15
Mlb349	*Coniothyrium* sp.	14
Mlb338	*Coniothyrium* sp.	12
Mlb39	*Coniothyrium* sp.	6
Mlb684	*Alternaria* sp.	69
Mlb682	*Alternaria* sp.	55
Mlb632	*Alternaria* sp.	51
Mlb568	*Alternaria* sp.	49
Mlb513	*Alternaria* sp.	48
Mlb129	*Alternaria* sp.	48
Mlb406	*Alternaria* sp.	47
Mlb305	*Alternaria* sp.	47
Mlb517	*Alternaria* sp.	45
Mlb104	*Alternaria* sp.	42
Mln930	*Phoma* sp.	48
Mln292	*Phoma* sp.	47
Mln808	*Phoma* sp.	36
Mln988	*Stemphylium* sp.	18
Mln992	*Stemphylium* sp.	18
Mlb641	*Stemphylium* sp.	17
Mln715	*Cadophora* sp.	72

DS, Disease severity, “Mild”, < 25% of diseased leaf area; “Low Moderate”, 26-50% of leaf area covered by disease; “Hight Moderate”, 51-75% of diseased leaf area; “Severe”, > 75% of diseased leaf area.after 6-week test period.

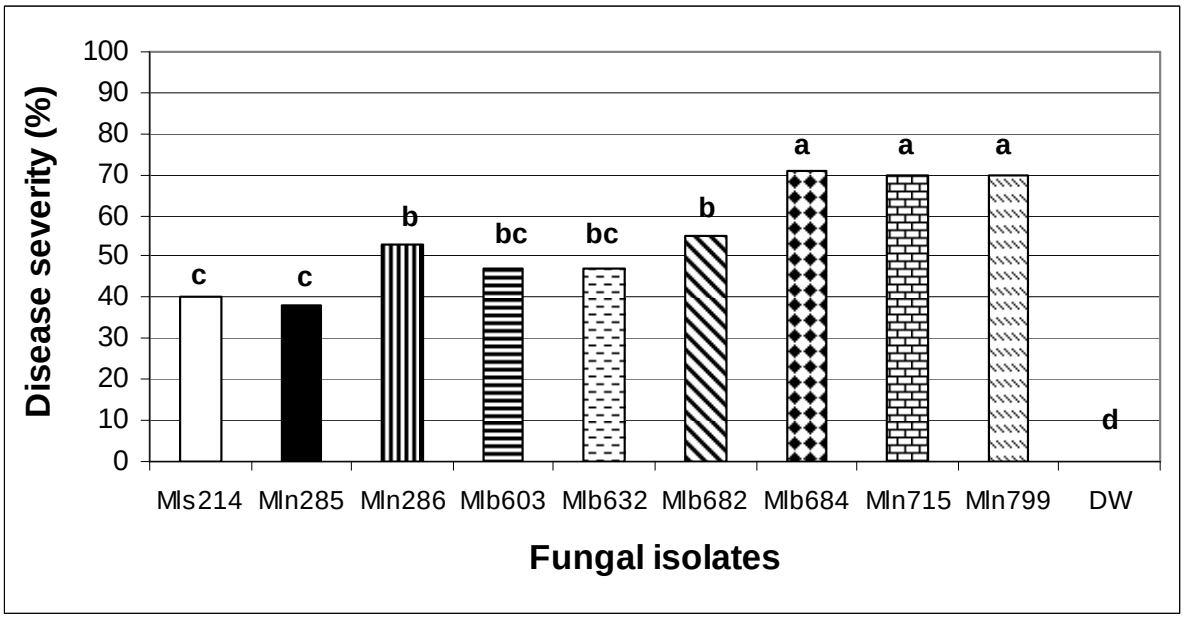
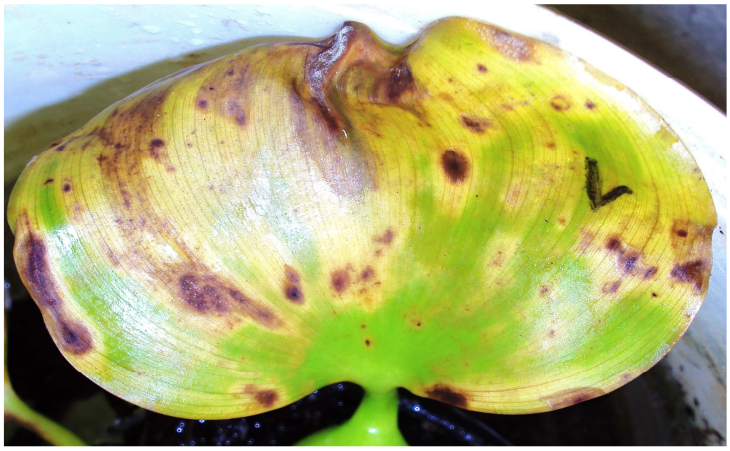


Figure 2. Disease severity (DS) recorded on water hyacinth plants 42-days post inoculation with nine fungal isolates (Mls214, Mln285, Mln286, Mlb603, Mlb632, Mlb682, Mlb684, Mln715 and Mln799). Duncan's multiple range test was used to separate means at $P<0.05$. Treatments having the same letters are not significantly different. DW: Sterlised water with no added pathogen.

A

B



C

Figure 3: Blight symptoms produced on water hyacinth plants by fungal without formulation in greenhouse, 85 ± 5% HR, 16 hours photoperiod. A. Leaf spots and blight symptoms after infection with isolate Mln799 (*Fusarium* sp.). B. Leaf spots and blight symptoms after infection with isolate Mln715 (*Cadophora* sp.). C. Leaf spots and blight symptoms after infection with isolate Mlb684 (*Alternaria* sp.).

II.4. Host range

Host range testing protocols using non-target plants have been developed to assess the safety of pathogens as biological agents (Babu et al., 2002). Among the fungi recorded to date as pathogens of water hyacinth, some of *Alternaria*, notably *A. eichhorniae* and *A. alternata*, have been proposed as possible promising biocontrol agents against this weed (Morsy 2004; Shabana 1997). Yet, Babu et al. (2003) report that carrot, sunflower and bean are susceptible to *A. eichhorniae*, and similar results have been reported for *A. alternata* (Morsy, 2004). This led us to test the host ranges of *C. malorum* isolate Mln715 and *Alternaria* sp. isolate Mlb684.

Table 4 shows the results obtained when these strains were delivered via water supplied to 19 crop-plants and weeds belonging to 15 different families. Four weeks after application of either isolate, water hyacinth (all three ecotypes) or *Salvinia* showed lesions on their leaves, whereas none of the other plants showed any signs of infection.

Our inoculation method could affect this outcome indeed, in field use the mycoherbicide will be direct spraying on the water hyacinth and *Salvinia*. Based on our results, these fungal isolates would not be expected to affect economically important plants in Mali, and should thus be viewed as potential candidates for managing water hyacinth infestations.

Table 4. Plants used in host-specificity test of isolates Mlb684 (*Alternaria* sp.) and Mln715 (*Cadophora* sp.) (+ means shows infection).

Family	Common name	Rating for Mlb684	Mln715
Rosaceae	Pear	-	-
	Apple	-	-
Poaceae	Rice	-	-
	Sorghum	-	-
	Corn	-	-
Amaranthaceae	Beet	-	-
	Amaranth	-	-
Apiaceae	Carrot	-	-
Caricaceae	Papaya	-	-
Liliaceae	Onion	-	-
Brassicaceae	Turnip	-	-
Fabaceae	Bean	-	-
Asteraceae	Sunflower	-	-
Malvaceae	Sorrel of Guinea	-	-
Piperaceae	Pepper	-	-
Apiaceae	Celery	-	-
Solanaceae	Tomato	-	-
Pontederiaceae	Water hyacinth		
	Ecotype 1	+	+
	Ecotype 2	+	+
	Ecotype 3	+	+
Salviniaceae	Aquatic fern	+	+

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CHAPTER III

EFFECT OF TEMPERATURE AND WATER ACTIVITY ON SPORE GERMINATION AND MYCELIAL GROWTH OF THREE FUNGAL BIOCONTROL AGENTS AGAINST WATER HYACINTH (*EICHHORNIA CRASSIPES*)

EFFECT OF TEMPERATURE AND WATER ACTIVITY ON SPORE GERMINATION AND MYCELIAL GROWTH OF THREE FUNGAL BIOCONTROL AGENTS AGAINST WATER HYACINTH (*EICHHORNIA CRASSIPES*)

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Abstract

Aims: To determine the effect of water activity (a_w = 0.880 - 0.960) and temperature (15-35°C) on the percentage of viable conidia and mycelial growth of three biocontrol agents effective against water hyacinth in Mali: *Alternaria* sp. isolate Mlb684, *Fusarium sacchari* isolate Mln799, and *Cadophora malorum* isolate Mln715.
Methods and results: The fungi were grown *in vitro* on plates containing PDA medium at different a_w values (glycerol being added to adjust the a_w). The percentage of viable conidia and radial growth rate decreased with decreasing water activity. Statistical analysis showed a significant effect of a_w, temperature, and the a_w x temperature interaction on mycelial growth (P<0.0001). Water activity emerged as the factor exerting the greatest influence. Differences were observed between the fungi tested, the *C. malorum* appearing more tolerant to low a_w and the *F. sacchari* more tolerant to high temperature (35°C). Growth models predicting the combined effect of a_w and temperature were developed and response surfaces generated, showing fairly good agreement with the experimental values.
Conclusion: Our results confirm the previous finding that a_w has a greater influence than temperature on fungal growth. Under most conditions, variation of environmental factors has a detrimental influence on the percentage of viable conidia and mycelial growth rate of fungal isolates.
Significance and impact of the study: The developed models may contribute to predicting the best environmental conditions for use of these fungi as effective biocontrol agents against water hyacinth.
Keywords: Temperature; water activity, percentage of viable conidia, growth rate; biocontrol agents; predictive models.
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III.1. Introduction

Water hyacinth (*Eichhornia crassipes*), originally from South America, is the most prolific aquatic weed worldwide. Since its introduction in the 1990's as an ornamental plant into aquatic areas in Mali, it has become a main focus of intense control efforts (Dagno, 2006).

Water hyacinth has become widespread and is viewed as the worst aquatic weed throughout the tropical and subtropical regions (El Morsy, 2004). The 'explosive' growth of the plant and its ability to infest a wide range of freshwater habitats has created enormous environmental and economical problems. Water hyacinth is considered a major aquatic weed in Africa (Niger, Benin, Congo, Egypt, Tanzania, Uganda, Mali, and South Africa), Europe (Spain), and several Asian and American countries (Dagno et al., 2007). It causes widespread problems for millions of users of water bodies and water resources, and this is especially the case in Mali. The Malian authority spends many million dollars each year to control water hyacinth infestation (Dagno, 2006). Several investigators have therefore focused on controlling water hyacinth by physical, chemical, or biological methods (Charudattan, 2005). Among the control methods available, biological control is increasingly viewed as an attractive, eco-friendly method for use in agriculture (Lahlali and Hijri, 2010).

Biocontrol by means of microorganisms is an emerging strategy in many countries infested with water hyacinth. Several good arguments suggest that plant pathogens are worth consideration as biocontrol agents: pathogens can cause significant reductions in water hyacinth biomass, notably following natural disease after severe attacks by insects, or when used as inundative bioherbicides (Charudattan, 2001). Several previous findings highlight the potential of *Acremonium zonatum*, *Alternaria eichhorniae*, and *Cercospora piaropi* for controlling water hyacinth (i.e. reducing the weed's biomass) under controlled conditions (Shabana and Mohamed, 2005). In Mali, research on the biological control of water hyacinth by means microorganisms was initiated in 2006. It has led to the isolation and identification of *Alternaria* sp. (isolate Mlb684), *F. sacchari* (isolate Mln799), and *Cadophora malorum* (isolate Mln715) (Dagno et al., unpublished data). Currently there are no data available on large-scale trials carried out with these fungal isolates, used as aqueous conidial preparations.

The variability of the performance of these isolates as biocontrol agents may be influenced by environmental factors that vary over time and from one area to another. Nanguy et al. (2010), Begoude et al. (2007), Plaza et al. (2003), and Patriarca et al. (2001) reported that water activity (a_w) and temperature are the principal abiotic parameters determining the germination and fungal growth. Accordingly, knowledge of the biology of biocontrol agents against water hyacinth, and notably regarding their germination and growth in relation to temperature and a_w, should be useful in developing a more effective mycoherbicide. Boyette et al. (2007) reported that these two environmental factors are the most important parameters influencing the efficacy of a mycoherbicide.

To optimize the practical use of a biological control agent, it is essential to understand how the physical environment affects the agent's survival, germination, and growth (Sanogo et al., 2002). Response surface methodology (RSM) is the approach most often used to model relationships between a combination of factors and an organism's growth curve parameters (Devlieghere et al. 1998).

To our knowledge, no report is available on the effects of temperature and water activity on the development of *F. sacchari*, or *C. malorum*. Regarding *Alternaria*, Pose et al. (2009) reported the increase of the germination and the growth rate of *Alternaria alternata* with increasing a_w values of substrate. They observed no growth or germination at the lowest a_w level evaluated (0.904) after 100 days of incubation at 6 °C and 15 °C. With the exception of *F. sacchari*, none of these fungi pose any risk to human, animal health or economically important crops in Mali and are viewed as attractive candidates for managing water hyacinth infestations in Mali.

Hence, the aim of the present work was to assess the effects of temperature and a_w on the percentage of viable conidia and mycelial growth of *F. sacchari*, *C. Malorum* and *Alternaria* sp. and to elaborate predictive models based on the collected mycelial growth data.

III.2. Materials and Methods

III.2.1. Fungi

Fusarium sacchari isolate Mln799, *Cadophora malorum* isolate Mln715, and *Alternaria* sp. isolate Mlb684 were identified by the Industrial Fungal & Yeast Collection (BCCM/MUCL- Louvain-la-Neuve, Belgium) and by Dr. E.G. Simmons (USA). For long-term storage, the strains were placed at -70°C in tubes containing 25% glycerol at the Plant Pathology Unit (Gembloux Agro-Bio Tech, University of Liege). The initial conidial inocula used in the experiments were taken from Petri-dish cultures on Potato Dextrose Agar (PDA, Merck, Darmstadt, Germany), preserved at 4°C for no more than 6 months, and then subcultured at 25°C on different culture media before use.

III.2.2. Media

One specific medium was used for each fungal isolate, PDA for *F. sacchari*, V8 agar for *Alternaria* sp., and MA2 (Malt agar 20%) for *C. malorum*. The water activity was adjusted by addition of increasing amounts of glycerol to obtain levels of 0.960, 0.920, and 0.880 at 15, 25, and 35°C (Lahlali et al. 2008). The range of temperature and a_w were chosen according to their minimum and maximum averages recorded in Mali. The a_w of all media was measured with an AquaLab 3TE (Decagon Device, Inc. 2365 NE Hopkins Court Pullman, WA 99163 USA).

III.2.3. Effect of temperature and a_w on the percentage of viable conidia and mycelial growth of fungal

The effects of a_w and temperature on the percentage of viable conidia were studied for *F. sacchari*, *C. malorum* and *Alternaria* sp. Percentage of viable conidia was evaluated at three a_w values and three temperatures 4, 8, and 24 h after inoculation of Petri dishes containing the test media. For each a_w-temperature combination there were three Petri dishes and each Petri dish was seeded with three individual 10-µl droplets (containing 1×10^5 spores ml^{-1}) of conidial suspension in separate wells. After inoculation, the Petri plates were sealed in polyethylene bags to prevent water loss and placed immediately in incubators set at the appropriate temperature. The preservation of water content in the media were checked by measuring the a_w of inoculated Petri dishes at the end of experiment and no change in the a_w of any tested medium was detected. At each assessment time, the percentage of viable conidia was estimated by observation under the microscope (at 40x or 100x magnification) of 100 conidia from each droplet of inoculum, thus yielding a total of nine counts per treatment at each time (Xu et al., 2001).

The spores were considered alive when the length of the germinate tube was equal to half of the diameter of the spore (Paul et al., 1992). To evaluate the effects on radial growth, a 10 µl aliquot of 10 µl 10^5 spores ml^{-1} was inoculated at the centre of Petri dishes containing a test medium. Petri plates were sealed and then incubated at each temperature.

The average radial growth of each growing mycelial colony was measured daily (in mm) in two perpendicular directions without opening the Petri dishes, until the plates were completely colonized (Marin et al., 1996). Growth rates (mm day^{-1}) were calculated for each a_w-temperature combination by linear regression from the linear phase of the growth curve. This experiment was conducted three times with three replicates.

III.2.4. Statistical analyses

A fully factorial design run in triplicate was used to generate the percentage of viable conidia and growth rate of *F. sacchari* (isolate Mln799), *C. malorum* (isolate Mln715) and *Alternaria* sp. (isolate Mlb684) in modified media at three temperatures and three a_w levels. Variance analysis was used to assess the effects of temperature and a_w on the percentage of viable conidia and mycelial growth *in vitro*. Growth rates were subjected to the general linear model procedure of the Statistical Analysis System (SAS software version 9.1. Cary, NC, USA). All statistical significances were estimated at $P = 0.05$. Where ANOVA revealed significant differences, Duncan's multiple range tests were applied to the means. Percentages of viable conidia were modelled using a nonlinear equation $y = ax^2 + bx + c$, where y, x, (a and b), and c represent respectively the percentage of viable conidia, incubation temperature, model parameters and the response value of y for all factors equal to zero.

MINITAB – 15 ENGLISH was used to apply Response Surface Methodology (RSM) to a 3^2 factorial design. Temperature (15, 25, and 35°C) and a_w (0.880; 0.920, and 0.960) were the studied factors, and the design included 9 experiments with three replicates. The following quadratic polynomial model was fitted to the response:

$$Y = B_0 + \sum_{i=1}^{2} B_i X_i + \sum_{i=1}^{2} B_{ii} X_i^2 + \sum_{i=1}^{2} \sum_{j=i+1} B_{ij} X_i X_j$$

where Y is the response (growth rate in mm day^{-1}), B_0 is a constant coefficient, X_i are coded variables that can have three values (-1, 0, or 1), B_i are linear coefficients, B_{ij} are the second-order interaction coefficients, and B_{ii} are the quadratic coefficients. All model coefficient values were calculated by multiple regression analysis. Interpretation of the data was based on the sign (positive or negative effect on the response) and statistical significance ($P < 0.05$) of each coefficient. The notion of major effects $\beta i.Xi$ and $\beta j.Xj$, assumes that the main effects are both positive and the interaction effect $\beta ij.Xi.Xj$ is antagonistic (negative) or synergistic (positive). R^2 (the coefficient of determination) and the adjusted R^2 were employed to evaluate regression model performance.

III.3. Results

III.3.1. Effects of temperature and a_w on percentage of viable conidia.

Figure 1 shows, for each isolate and at different incubation times, how the percentage of viable conidia (i.e. the percentage of conidia having germinated) varied at different water activities as a function of the incubation temperature. As expected, the percentage of viable conidia increased over time. At $a_w = 0.88$, all three isolates germinated poorly, *Alternaria* sp. showing the lowest rates (17% after 24h at 25°C) and *F. sacchari* the highest (52% after 24 h at 35°C). Overall, the percentage of viable conidia was found to improve with increasing water activity, reaching or approaching 100%, at the considered strain's optimal germination temperature, within 24 h at $a_w = 0.96$ and $a_w = 0.92$. The *C. malorum* and *Alternaria* sp. both germinated better at 25°C than at 15°C or 35°C, but *F. sacchari* seemed, under most conditions, to germinate best at 35°C. ANOVA showed the main effects of temperature and a_w on the percentage of viable conidia to be significant ($P < 0.01$) (data not shown).

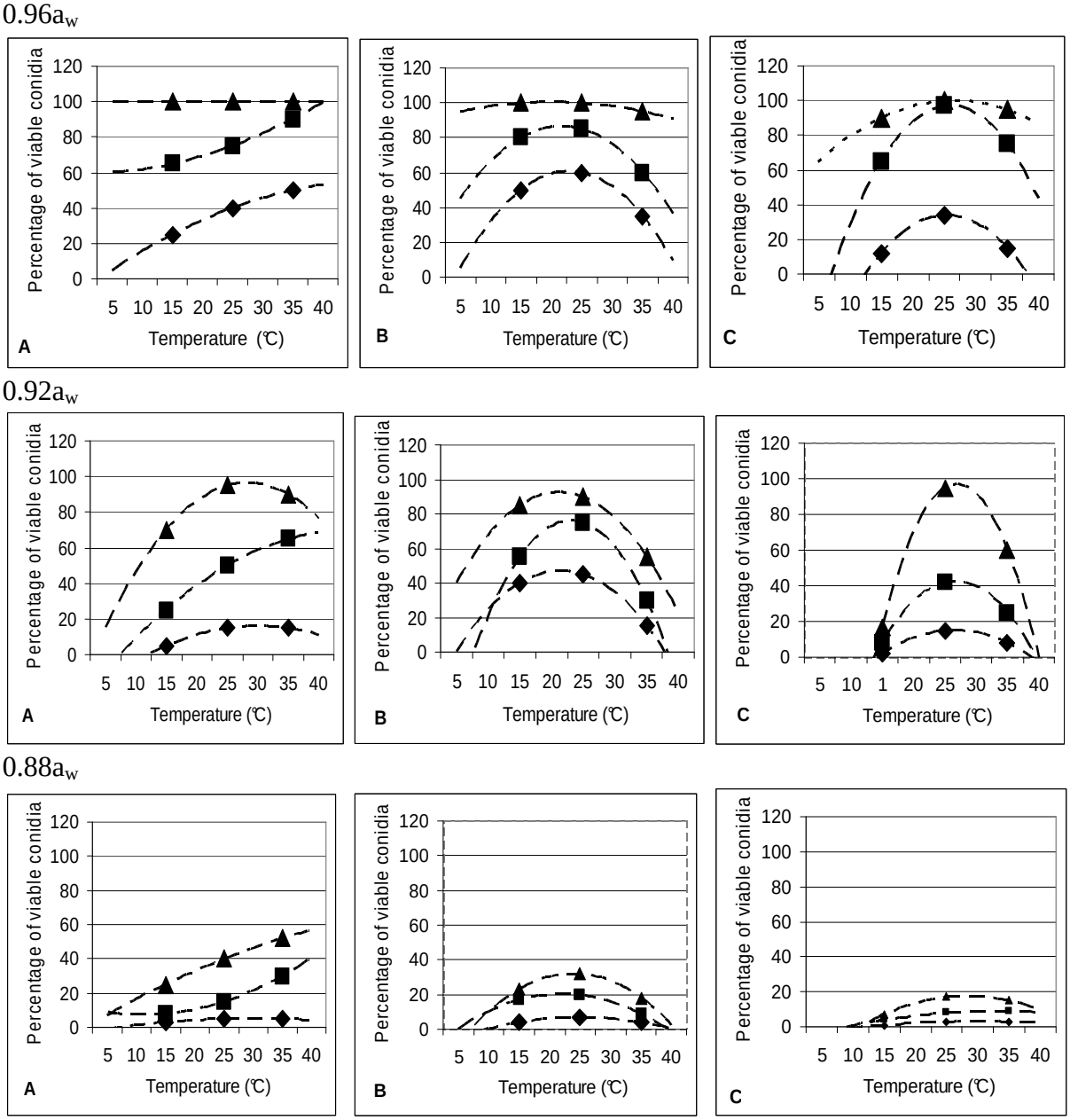

Figure 1: Mean germination rate as a function of temperature according to the strain, the water activity, and the incubation time. (A) *Fusarium sacchari* (isolate Mln799), (B) *Cadophora malorum* (isolate Mln715), (C) *Alternaria* sp. (isolate Mlb684). The symbols ♦, ■, and ▲ indicate the germination time (respectively 4, 8, and 24 h). The dotted lines are fitted curves (equation in the text).

III.3.2. Effects of temperature and water activity on mycelial growth

Figure 2 presents the average radial growth rates (in mm day^{-1}) of the three strains under the conditions tested, along with the results of Duncan's multiple range analysis showing which growth-rate differences were statistically significant. *Alternaria* sp. and *F. sacchari* showed no growth at a_w = 0.88 at any temperature (Figures 2A and 2C), but the *C. malorum* did show some growth at this a_w (Figure 2B). All three isolates were found to grow faster with increasing water activity. For *C. malorum*, an increase was observed only at a_w =

0.96 (Figure 2B). When growth was observed, it was always better at 25°C than at 15°C or 35°C, although in one case (*C. malorum* at $a_w = 0.96$, Figure 2B), the difference between 15°C and 25°C was not significant. The *Alternaria* sp. (isolate Mlb684) failed to grow at 35°C at any a_w (Figure 2A). The highest growth rate (3.5 mm day^{-1}) was observed for *F. sacchari* at $a_w = 0.96$ and 25°C (Figure 2C). Variance analysis (Table 1) showed that temperature, a_w, and their interaction significantly influenced the fungal growth rate.

Table 1 Variance analysis of the effects of water activity (a_w), temperature (T), and their interactions on the growth rate of *Alternaria* sp. (isolate Mlb684), *Cadophora malorum* (isolate Mln715), and *Fusarium sacchari* (isolate Mln799).

Isolate	Source of variation	*df*	MS	*F* statistic	Pr > *F*
Mlb684	a_w	2	0.008255	18.71	0.0001**
	T°C	6	0.0042	20.15	0.0001**
	a_w x T°C	1	0.003997	9.06	0.0001**
Mln715	a_w	2	0.004130	36.75	0.0001**
	T°C	6	0.004250	35.60	0.0001**
	a_w x T°C	1	0.000234	2.08	0.0001**
Mln799	a_w	2	0.176814	190.62	0.0001**
	T°C	6	0.164250	213.60	0.0001**
	a_w x T°C	1	0.040472	43.63	0.0001**

MS, mean square; *df*, degrees of freedom; Pr, probability; ** significant ($P<0.0001$)

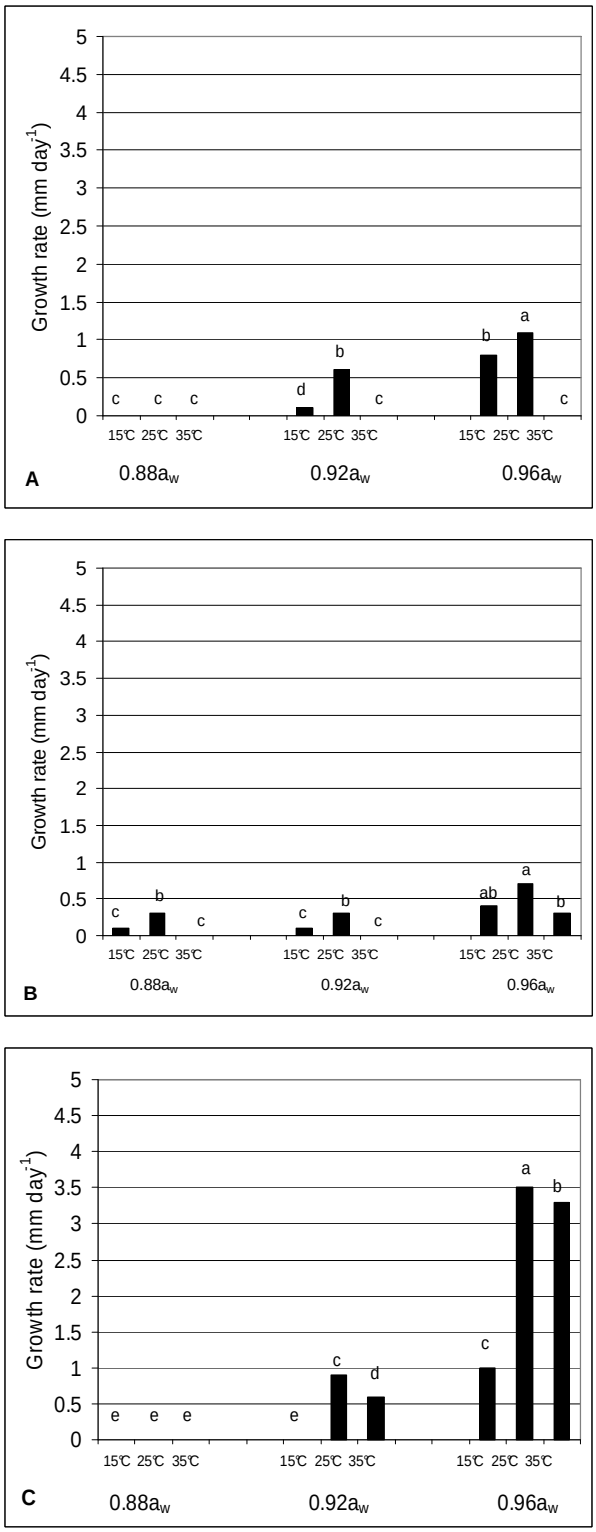

Figure 2: Average growth rates of (A) *Alternaria* sp. (isolate Mlb684), (B) *Cadophora malorum* (isolate Mln715), and (C) *Fusarium sacchari* (isolate Mln799) at different temperatures and water activities. Significance was determined by Duncan's multiple range tests. Results with the same letter are not significantly different (P<0.05).

III.3.3. Modelling the combined effect of temperature and a_w on the growth rates of fungal isolates.

RSM was then used to model the effects of temperature and water activity on the growth rate of our three potential biocontrol agents against water hyacinth. For each strain, a quadratic polynomial model based on a 3^2 factorial design was fitted to the data by multiple regression analysis (see Materials and Methods). Table 2 shows the respective R^2 values, 85.89%, 77.31%, and 93.40% for *C. malorum* (isolate Mln715), *Alternaria* sp. (isolate Mlb684), and *F. sacchari* (isolate Mln799) respectively, the determined model coefficients, and their levels of significance. The corresponding response surfaces (Figure 3) clearly show a strong influence of both temperature and a_w on the growth of all three strains. For the temperature and water activities tested, there is fairly good agreement between the observed and predicted values (Table 3).

Table 2. Coefficients of the models describing the growth rates of *Cadophora malorum* (isolate Mln715), *Alternaria* sp. (isolate Mlb684), and *Fusarium sacchari* (isolate Mln799) at different temperatures (T) and water activity (a_w), and significance thereof.

Factor or interaction	Coefficient	Mln715	Mlb684	Mln799
	R^2	85.89	77.31	93.40
	β0	0.044**	0.054**	0.103**
a_w	β1	0.017**	0.033**	0.130**
T	β2	-0.012**	-0.014*	0.050**
a_w^2	β11	0.014*	0.010^{ns}	0.077**
T^2	β22	-0.037**	-0.046**	-0.070**
a_w x T	β12	-0.004^{ns}	-0.018**	-0.070**

ns = not significant (P>0.05), * = significant (P<0.05), ** = highly significant (0.05<P<0.0001)

Table 3. Experimental and predicted values of the growth rate for *Fusarium sacchari* (isolate Mln799), *Cadophora malorum* (isolate Mln715), and *Alternaria* sp. isolate Mlb684. To obtain the predicted values, a factorial design (3^2) was applied, the factors studied being temperature (T) and water activity (a_w).

Experimental factors					Radial growth rate (mm day^{-1})					
Experimental value			Coded value		Observed value			Predicted value		
	a_w	T	a_w	T	Mln799	Mln715	Mlb684	Mln799	Mln715	Mlb684
E1	0.96	15	1	-1	1.00±0.00	0.40±0.00	0.80±0.01	1.30	0.50	0.80
E2	0.96	25	1	0	3.50±0.00	0.70±0.00	1.10±0.00	3.10	0.70	0.90
E3	0.96	35	1	1	3.30±0.00	0.30±0.00	0.00±0.00	3.50	0.20	0.10
E4	0.92	15	0	-1	0.00±0.00	0.10±0.00	0.10±0.00	0.00	0.10	0.20
E5	0.92	25	0	0	0.90±0.00	0.30±0.00	0.60±0.01	1.00	0.40	0.50
E6	0.92	35	0	1	0.60±0.01	0.00±0.00	0.00±0.00	0.80	0.00	0.00
E7	0.88	15	-1	-1	0.00±0.00	0.10±0.00	0.00±0.00	0.00	0.10	0.00
E8	0.88	25	-1	0	0.00±0.00	0.30±0.00	0.00±0.00	0.50	0.40	0.30
E9	0.88	35	-1	1	0.00±0.00	0.00±0.00	0.00±0.00	0.00	0.00	0.00

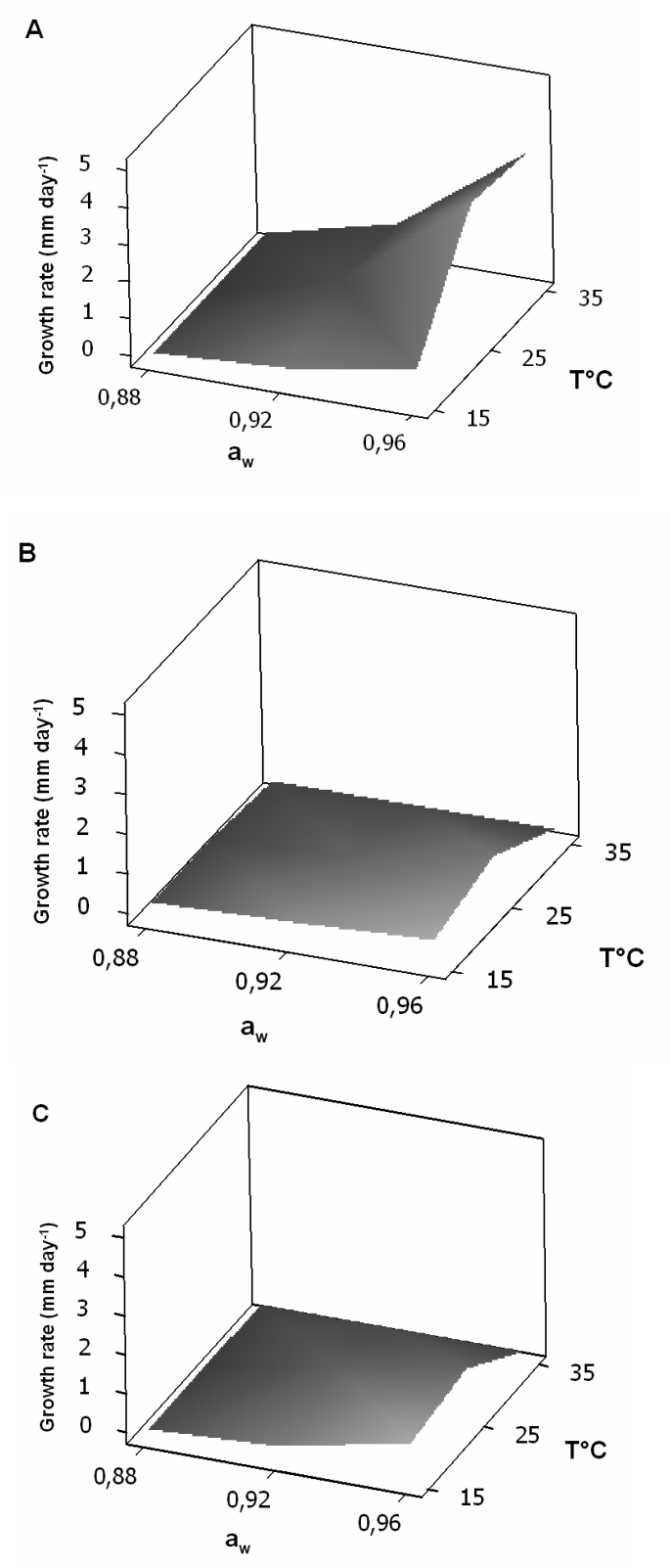

Figure 3: Response surfaces representing the predicted effects of water activity and temperature on the growth rate of (A) *Fusarium sacchari* (isolate Mln799), (B) *Cadophora malorum* (isolate Mln715), and (C) *Alternaria* sp. (isolate Mlb684).

III.4. Discussion

We have focused here on the percentage of viable conidia and mycelial growth rates of three fungal pathogens, *F. sacchari*, *C. malorum*, and *Alternaria* sp., as possible indicators of the capacity of these pathogens to colonize the water hyacinth ecosystem. In greenhouse trials, these organisms have previously been found to induce 70% (isolates Mln799, Mln715) or 71% (isolate Mlb684) foliar lesions in this plant. Yet the efficacy of such pathogens tends to be greater and less variable in the greenhouse than in the field (Boyette et al., 2007), and environmental factors are believed to influence importantly the efficacy of weed biocontrol agents under field conditions. Temperature and water activity are suspected of being the major determining factors (Babu et al., 2003; Charudattan 2005; Nanguy et al., 2010), but with the exception of *Alternaria* (Pose et al., 2009) we have found no report in the literature describing the influence of these factors on the growth or germination of the above-mentioned strains, We have therefore studied the behaviour of these fungi *in vitro* on glycerol-supplemented media and modelled their growth.

In the present study, glycerol was the only solute used to adjust the a_w of the medium. This solute can support growth because it is a potential carbon source for microorganisms (Parra et al., 2004). In addition, glycerol exhibits no inhibitory effect (Baxter et al., 1998) and can be used to reach lower a_w values than other solutes such as NaCl (Lahlali et al., 2005).

From our experiments, water activity emerged as a crucial determinant of germination for all three strains, their germination being fastest at $a_w = 0.96$ (reaching 100% at 25°C within 24 h) and slowest at $a_w = 0.88$. At high water activity, their conidia could germinate fast over a wide range of temperatures, from 15 to 35°C. At $a_w = 0.96$ and 25°C, for example, it took only 4 h for 35-60% of the viable conidia to germinate, depending on the organism studied. This may explain why long wetness periods do not hinder infection of water hyacinth plant by these three fungal pathogens. Our germination data are in agreement with results previously reported for two apple brown rot fungi, *Monilinia fructigena* and *M. fructicola* (Tamm and Fluckinger, 1993; Xu et al., 2001). Pose et al. (2009) reported that the germination time of *A. alternata* increased with a reduction on a_w.

Our growth data show that temperature and a_w are key determinants of growth for the studied strains. Water activity emerges as the factor having the greatest influence on mycelial growth of fungal isolates. No growth was observed at a_w of 0.88 for both isolates *Alternaria* sp. and *F. sacchari* regardless of temperature while there was low growth for *C. malorum* at 15 and 25°C. Our results demonstrated that a_w has a greater influence than temperature on the growth rates of fungal isolates Mlb684, Mln715 and Mln799. These results are in agreement with the previous finding that the amount of available water in the substrate and the surrounding environment is very important for fungal growth (Lahlali et al., 2008). Furthermore, in accordance with Sparringa et al. (2002) on *Rhizopus oligosporus*, a significant interaction between the two studied factors was shown.

Both germination and growth are important for the efficacy of a fungus used as a biocontrol agent. Of the three organisms studied, both the *F. sacchari* and the *C. malorum* isolates appear to germinate better at $a_w = 0.88$ than the *Alternaria* sp. *F. sacchari*, moreover, shows better germination at temperatures above 35°C than either of the other strains, whose temperature optimum for germination is near 25°C. Pose et al. (2009) reported similar patterns of the influence of a_w and temperature on the germination and the growth rate of *A. alternata* with shortest germination at 35°C and no growth at $a_w = 0.90$ and temperature of

15°C. Regarding growth, the *F. sacchari* again shows better tolerance of higher temperature, whereas the *C. malorum* shows better tolerance of water stress. Our findings suggest that countering water stress could be a means of improving the performance of fungal biocontrol agents under field conditions. Vegetable oil might be used for this purpose (Shabana, 2004).

Data are available concerning the effects of temperature and water activity on a number of other fungi. Lasram et al. (2010) report optimal growth of *Aspergillus carbonarius* at temperatures ranging from 25 to 30°C and $a_w = 0.99$. They mention that this fungus grows poorly at 15°C and at $a_w \leq 0.90$. Romero et al. (2010) likewise report slow growth of *A. carbonarius* at low a_w and temperature (0.83 and 15°C). Similar effects of a_w and temperature were observed by Bekada et al. (2008) for the fungus *Mucor racemosus*, and Begoude et al. (2007) found that *Trichoderma asperellum* failed to grow at $a_w = 0.88$, whatever the temperature.

On the basis of our radial growth data, we have developed models as tools for interpreting such data. Within the temperature and a_w ranges specified, the selected models predict fairly accurately the growth rates of these three strains. At $a_w = 0.88$, for instance, the *C. malorum* model correctly predicts slight growth at 15 and 25°C but none at 35°C, and the *F. sacchari* and *Alternaria* sp. models correctly predict no growth of either strain at 15 or 35°C. Yet there are some slight discrepancies between the observed and predicted values, such as the slight growth predicted for *F. sacchari* and *Alternaria* sp. at $a_w = 0.88$ for 25°C.

Modelling the growth of these fungal isolates on a solid substrate is a first step towards simulating what happens when these biocontrol agents are applied to water hyacinth, extrapolating their behaviours to field conditions, and finding a formulation that takes into account their ecophysiological traits. Yet it is crucial to emphasize that our glycerol models for *F. sacchari*, *C. malorum*, and *Alternaria* sp. are based on data obtained under *in vitro* conditions. Our models might overestimate growth under natural conditions, because our strains were grown on a nutrient-rich artificial medium under good light conditions. Furthermore, environmental factors other than those studied here may be involved, such as relative humidity, UV, pH, and interactions with organisms of the microflora present on the leaf surface of water hyacinth.

It may thus be mandatory to develop models based on *in vivo* conditions and taking into account the factors just mentioned. In this framework, it might be possible to integrate models such as ours into a broader study of the impact of environmental factors on the biocontrol agent - weed system studied here. Good models of fungal behaviour under field conditions could provide a basis for a more rational control strategy, possibly involving the use of a formulation protecting *F. sacchari*, *C. malorum*, and *Alternaria* sp. isolate Mlb684 against unfavourable environmental factors.

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CHAPTER IV

ALTERNARIA JACINTHICOLA, A NEW FUNGAL SPECIES CAUSING BLIGHT LEAF DISEASE ON WATER HYACINTH (*EICHHORNIA CRASSIPES*)

ALTERNARIA JACINTHICOLA, A NEW FUNGAL SPECIES CAUSING BLIGHT LEAF DISEASE ON WATER HYACINTH (*EICHHORNIA CRASSIPES*)

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Abstract

Water hyacinth (*Eichhornia crassipes*) is environmental, agriculture and healthy problems in Mali. This is particularly severe in the District of Bamako and the irrigation systems of the "Office du Niger" area. Several million dollars are spent each year by the "Office du Niger" and "Energie du Mali" to control this weed in the River Niger. *Alternaria* sp. isolate Mlb684 was isolated from diseased water hyacinth plant, in the frame of a 2 years survey for fungal pathogens of water hyacinth in Mali. This fungal isolate was identified as a potential mycoherbicide for sustainable management of water hyacinth. The aim of this study was to characterize this *Alternaria* sp. isolate Mlb684 in order to classify it among the *Alternaria* genus. The characterization was based on a morphological description and a DNA sequence analyse. Various genes amplified from *Alternaria* sp. isolate Mlb684 were compared to those existing in Genbank. These genes were 18S ribosomal rDNA gene, ITS rDNA gene, elongation factor-1 alpha (EF1a) gene, calmodulin gene and actin gene. DNA sequence comparisons and morphological description provided enough evidence *Alternaria* sp. isolate Mlb684 belonged to the *Alternaria* genus and was distinct enough from any other known *Alternaria* species. Based on this evidence, the new *Alternaria* sp. isolate Mlb684 was called "*Alternaria jacinthicola* Dagno & M.H. Jijakli". A specimen culture has been deposited in the Industrial Fungal & Yeast Collection (BCCM/MUCL, Belgium) under the accession number: MUCL 53159 and all DNA sequences were deposited in GenBank (NCBI).

Key words: 18S rDNA, actin, calmodulin, *Alternaria jacinthicola, Eichhornia crassipes*, elongation factor 1 alpha, genetic characterization, ITS rDNA, water hyacinth.

IV.1. Introduction

Water hyacinth (*Eichhornia crassipes*) has spread throughout Africa causing widespread problems to millions of users of water bodies and water resources. The plant affects irrigation, water flow, water use, and navigation; it also poses a health risk by enabling the breeding of mosquitos, bilharzias, and other human parasites (Dagno et al., 2007).

Biocontrol has been considered as the most adequate control strategy against water hyacinth (Charudattan, 2005). Among possibilities offered by the biocontrol management, fungal pathogens could be an efficient control tool against this aquatic weed. Several research groups have identified promising microbial fungal agents notably *Alternaria eichhorniae* Nag Raj & Ponnappa and *A. Alternata* (Fr.) Keissler that might be developped and used as mycoherbicides (El-Morsy, 2006; Babu et al., 2002; Shabana, 1997).

Research on fungal pathogens of water hyacinth began in 2006 in Mali and led to the isolation and identification of *Gibberella sacchari* Summerell & J.F. Leslie (isolate Mln799), *Cadophora malorum* (Kidd & Beaumont) W. Grams (isolate Mln715) and isolate Mlb684 (Dagno et al., 2011a). Among the 3 selected fungi, an unusual fungus, isolate Mlb684 applied in unrefined *Carapa procera* (L) oil and refined palm oils caused 87 to 90% of disease severity on water hyacinth 6 weeks after treatment respectively (Dagno et al., 2011b).

In 2007, the Industrial Fungal & Yeast Collection (BCCM/MUCL, Belgium) identified isolate Mlb684 as *Alternaria* sp. with reference BCCM/MUCL DIV/07-119C. However, morphological characters displayed by this isolate including the pattern and sporulation structure of this isolate hardly match with those currently used to describe known species in the genus *Alternaria* (E.G.S. characteristics, as described in the literature: Simmons, 2004; Simmons, 1999; Simmons and Roberts, 1993. It was therefore assumed that this isolate could belongs to a novel species of *Alternaria*.

Two *Alternaria* species were until now reported on water hyacinth. *A. eichhorniae* and *A. alternata* were recognized as virulent pathogens on *Eichhornia crassipes* species. They are best known as the causal agent of leaf blight disease on water hyacinth in Egypt and India (Shabana et al., 1995; Aneja and Sing, 1989).

The present study was designed to provide a taxonomic position of the isolate Mlb684 at the species level, using morphological characterization and DNA sequence comparisons.

IV.2. Materials and methods

IV.2.1. Fungal isolation and specimen collection

Infected parts of water hyacinth (petiole and leaf) were collected from the river Niger in the District of Bamako, the lake of Sebougou in Segou, and the central collector of Niono with GPS coordinates "12° 40' N, 7° 59' W" ; "13° 26' N, 6° 15' W" and "14° 15' N, 5° 59' W" respectively. Samples in clean plastic bags were brought to the laboratory and then stored at 4°C until examined.

Stored plant parts were scrubbed under running water to remove surface debris, dissected into small segments (approximately 1 × 1 cm), and surface-sterilized by sequential immersion in 96% ethanol for 30 seconds and then in 14% hypochlorite for 30 seconds. The segments were rinsed in sterilised water for 1 min. Surface-sterilised segments (4 segments/plate) were plated on Potato Dextrose Agar (PDA, Merck, Darmstadt, Germany) supplemented with 5 ppm streptomycin. Three plates were used for petiole and limb of water hyacinth plant. The plates were then incubated at 25°C for 5 to 7 days. Emergent fungi on the plant fragments were isolated and pure cultures were obtained by the single-spore technique, Fungi were then preserved at 4°C, for no more than 6 months, before use.

Isolate Mlb684 was selected during a previous assay conducted to identify potential mycoherbicide for water hyacinth (Dagno et al., 2011a,b). Pure cultures were deposited in the Culture Collection of the Phytopathology Unit, Gembloux Agro Bio Tech (GxABT), University of Liege, Belgium. Duplicates of key isolates (specimens) were also deposited to the Industrial Fungal & Yeast Collection (BCCM^{TM}/MUCL - Louvain-la-Neuve) Belgium.

For mass production, isolate Mlb684 was incubated on V8 agar during 2 weeks at 25°C and 16 hours photoperiod.

IV.2.2. Molecular analysis

A liquid culture from the isolate Mlb684 was performed in 100 ml of Potato Dextrose Broth (PDB) in a 250 ml erlenmeyer for 5 days. Pure DNA was obtained from the resulting culture using a quick DNA extraction method (Lefort and Douglas, 1999). Universal oligonucleotide primers (Table 1) targeting 5 fungal genes were used for PCR amplification. Primers ITS1, ITS4 and ITS5 (White et al., 1990) for the 18S and 28S rDNA sequence; primers NS1 and NS2 (White et al., 1990) for a partial 18S rDNA sequence; primers EF1-728F (Carbone and Kohn, 1999) and TEF1LLEre (Jaklitsch et al., 2005) for the elongation factor-1 alpha (EF1a); primers CAL-228 and CAL-737R (Carbone & Kohn, 1999) for calmodulin gene; and primers ACT-783R and ACT-512F (Carbone and Kohn, 1999) for actin gene.

PCR was carried out using the KAPA2G Robust PCR (KappaBiosystems, Japan) and each PCR amplification was performed in 20 µl reaction mixture consisting of 10 µl Maxima Sybr Green qPCR Master Mix 2X (Fermentas), 2 µl each of the forward and reverse primers (10 µM), 1 µl cDNA template (1ng/µl), and 5µl PCR-grade water. The cycling conditions were: pre-incubation for 10 min at 95°C, followed by 40 cycles, each consisting of 30s denaturing at 95°C, 40s annealing at 52°C, and 45s elongation at 72°C, the last cycle ending with a final 10-min extension at 72°C.

Total genomic DNA was extracted according to Lefort and Douglas (1999), and the concentration of the resulting DNA was determined with an ND-1000 UV/Vis spectrometer (NanoDrop Technologies, Wilmington, DE USA) version 3.1.0. Oligonucleotide primers (Table 1) were used to amplify and sequence the internal transcribed spacer (ITS) regions (including the partial 18 S and 28 S DNA genes) and regions corresponding to the genes encoding elongation factor-1 alpha (EF1a), calmodulin, and actin. DNA sequences were edited by Fasteris SA, Geneva, Switzerland (Lefort and Douglas, 1999). Resulting DNA sequences were deposited in GenBank (NCBI, Bethesda, MD, USA) and compared using the similarity search tool Blast.

DNA sequences recovered from GenBank for species close to the *Alternaria* isolate Mlb684 were aligned by ClustalW2 (Chenna et al., 2003) and used to generate molecular phylogenies with an optimal neighbour-joining method (Myers and Miller, 1988). Trees were then drawn with the software Jalview (Waterhouse et al., 2009); in addition, a Bayesian analysis was run to this one.

Table 1. PCR primers for amplification of genes of *Alternaria* sp. isolate Mlb684.

Primers names	Sequences
[1]ITS1	5'TCCGTAGGTGAACCTGCGG3'
[1]ITS4	5'TCCTCCGCTTATTGATATGC3'
[1]ITS5	5'GGAAGTAAAAGTCGTAACAAG3'
[1]NS1	5'GTAGTCATATGCTTGTCTC3'
[1]NS2	5'GGCTGCTGGCACCAG…..TGC3'
[2]EF1-728F	5'CATCGAGAAGTTCGAGAAGG3'
[3]TEF1LLErev	5'AATTTGCAGGCAATGTGG3'
[2]CAL-228	5'GAGTTCAAGGAGGCCTTCTCCC3'
[2]CAL-737R	5'CATCTTTCTGGCCATCATGG3'
[2]ACT-783R	5'TACGAGTCCTTCTGGCCCAT3'
[2]ACT-512F	5'ATGTGCAAGGCCGGTTTCGC3'

[1]White et al. (1990), [2]Carbone and Kohn (1999), [3]Jaklitsch et al. (2005)

IV.2.3. Morphology

Isolate Mlb684 were grown on V8 agar and potato-carrot agar mediums under strictly defined incubation conditions (Simmons, 1992) and examined for characteristics of the sporulation apparatus and conidium morphology to confirm species identity and compare morphological characters.

Morphological description was performed at 50x magnifications after 7-14 days old cultures. It was based on observations concerning colony growth, color, type of mycelia, size, and form arrangement of conidia. Identification key E.G.S. 00.000 was used to record conidia of this fungal isolate as describe by Simmons and Roberts, 1993.

IV.3. Results

IV.3.1. Molecular analysis

PCR resulted in the successful amplification of ITS rDNA (541 bp) gene using primers ITS1 and ITS4, 18S DNA (527 pb) gene with primers NS1 and NS2, elongation factor 1-alpha (1182 bp) gene with primers EF1-728F and TEF1LLErev, calmodulin (1182 bp) gene with primers CAL-228 and CAL-737R and actin (240 bp) gene with primers ACT-783R and ACT-512F for isolate Mlb684. All sequences determined in this study have been submitted to GenBank.

Based on DNA sequences of the 5 studied genes, sequence comparisons were performed between *Alternaria* sp. isolate Mlb684 and the closest species or isolates for which sequences were available. Comparison of the ITS rDNA gene showed that *Alternaria* sp. isolate Mlb684 was 100% identical to 3 other *Alternaria* isolates (Table 2). *Alternaria* sp. isolate MUCL 45333 is pathogen on wheat crop, in opposite, *Alternaria* sp. isolates IA2448 and IA249 that are reported infect regularly *Hylocereus undatus* fruits in Iran.

Concerning the 18S rDNA gene, *Alternaria* sp. isolate M1b684 showed 100% identity with 12 microorganisms among them 3 *Alternaria* species. *A. japonica* and *A. alternata* isolated from disease plants of *Brassica rapa* ssp. *oleifera*, indeed, *A. brassicicola* isolated from infected *Brassica oleracea* ssp. *capitata* in Canada. Similarly to the ITS rDNA gene, the 18S rDNA gene sequence of *Alternaria* sp. isolate M1b684 showed than a 98 to 99% identity with those of 72 other *Alternaria* or unknown cultured fungi isolates making.

Comparing elongation factor 1-alpha gene sequences yielded 2 related *Alternaria* species (Table 2). *Alternaria* sp. isolate CBS 174.52 showed 98% identity along 64% of its sequence with *Alternaria* sp. isolate M1b684 while *A. alternata* isolate AFTOL-ID 1610 shared 98% identity along 61% of its sequence *Alternaria* sp. isolate M1b684.

Comparing the calmodulin gene sequence of *Alternaria* sp. isolate M1b684 to GenBank sequences yielded no *Alternaria* species sequences and the closest microorganism was the *Pyrenophora tritici-repentis* isolate Pt-1C-BFP. This fungal species shared 97% identity over 42% of its sequence.

Finally when comparing the actin gene sequence of *Alternaria* sp. isolate M1b684 to GenBank sequences yielded 7 *Alternaria* species sequences ranging from 97% identity over 84% of its sequence to 91% identity along 83% of its sequence when compared. Table 2 illustrated these species. All isolates of *A. carotiincultae* and *A. radicina* were isolated from infected carrots.

An ITS sequence (GenBank accession EU314716) for the ITS rDNA gene of *A. eichhorniae* shared 97% of its sequence with the one of *Alternaria* sp. isolate M1b684 (GenBank accession HQ413695).

Fig.1A showed the genetic relationships between *Alternaria* sp. isolate M1b684 and the closest microorganisms for this elongation factor 1-alpha gene sequence, where it appears that it is quite distinct from the closest organisms. Genetic relationships are shown between closest calmodulin and actin gene sequences are shown respectively on fig.1B and fig.1C.

We have described phylogenetic relationships among *Alternaria* sp. isolate Mlb684 and *Alternaria* genera or isolate available in Genbank, based on sequences from five different genetic regions, ITS rDNA gene, 18S rDNA gene, EF1a gene, calmodulin gene and actin gene.

ITS rDNA sequence analysis of *Alternaria* sp. isolate M1b684 presented 99% of identity to those of 93 other fungi isolates. Indeed, the 18S rDNA gene sequence of same isolate M1b684 showed also than a 98 to 99% identity with those of 72 other *Alternaria* or unknown cultured fungi isolates.

In order to confirm the result obtained in phylogenies studies, a Bayesian analysis was run to this one (data not shown).

Table 2. Comparisons of DNA sequences were performed between *Alternaria* sp. isolate M1b684 and the closest fungal for which sequences were available in Genbank.

Fragment size	Species and sources	Genbank accession numbers	Homology percentage
ITS rDNA	*Alternaria* sp. (isolate MUCL 45333)	AY714488	100
	Alternaria sp. (isolate IA2448)	AY154699	100
	Alternaria sp. (isolate IA249)	AY154698	100
18S rDNA	*A. japonica*	U05199	100
	A. alternata	U05194	100
	A. brassicicola	U05197	100
EF1a	*Alternaria* sp. (isolate CBS 174.52)	DQ677911	98
	A. alternata (isolate AFTOL-ID 1610)	DQ677927	98
cmdA	*Pyrenophora tritici- repentis* (isolate Pt-1C-BFP)	XM00194109	97
act	*A. alternata*	GQ240307	97
	A. carotiincultae (isolates BMP0129)	EU141969	97
	A. carotiincultae (isolate BMP0095)	EU141972	97
	A. carotiincultae (isolate BMP0132)	EU141968	97
	A. radicina (isolate BMP0047)	EU141973	91
	A. radicina (isolate BMP0062)	EU141972	91
	A. radicina (isolate BMP0079)	EU141971	91

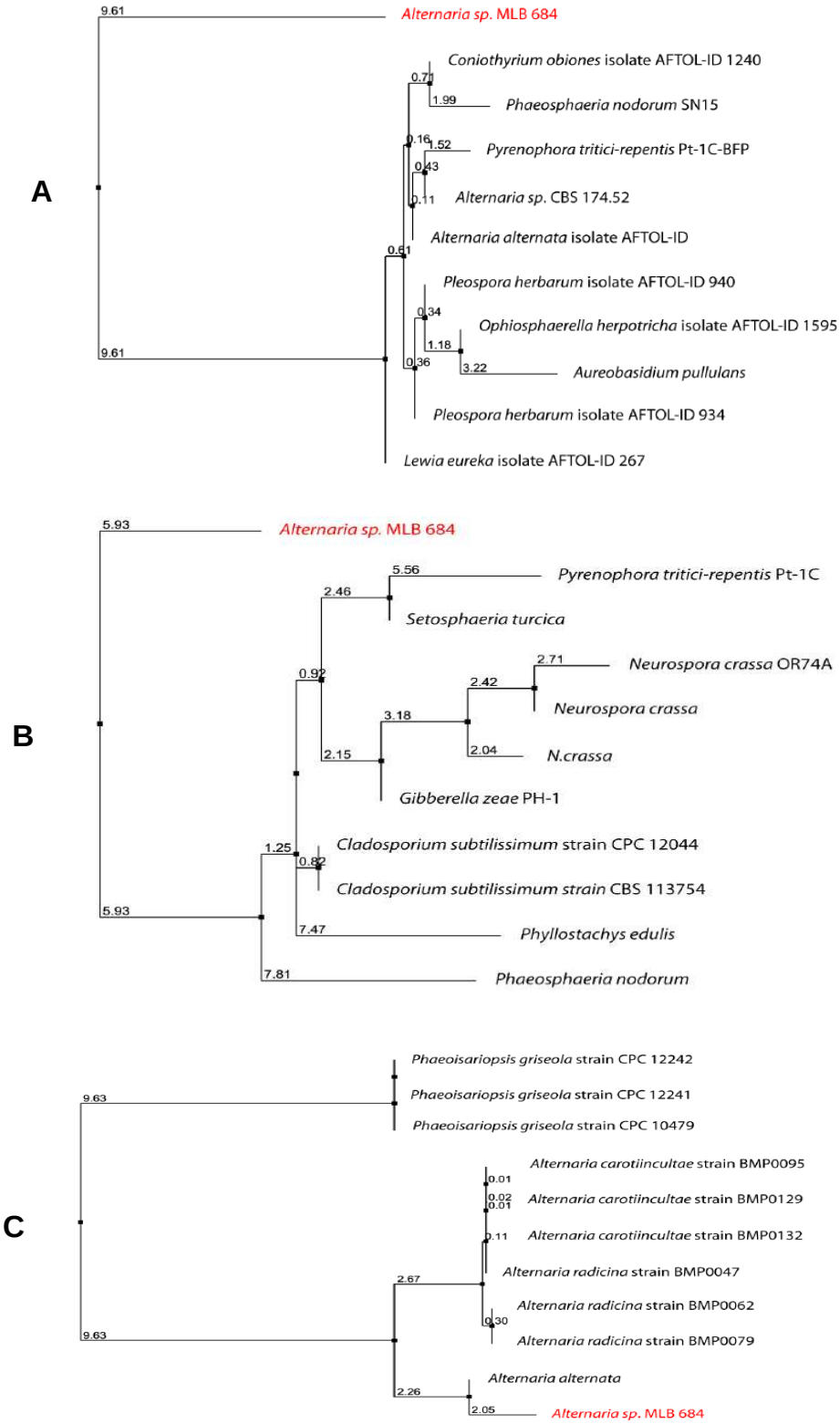

Figure 1. Molecular phylogenies of *Alternaria* sp. isolate Mlb684 based on 3 amplified and sequenced DNA regions. A) partial sequence of Elongation factor-1 alpha (EF1a) gene, B) partial sequence calmodulin gene, C) partial sequence of actin gene.

IV.3.2. Morphology

It produced an ash colony on V-8 agar and has well sporulated 2 weeks after incubation at 25°C with 16 photoperiod. Colony and conidia were easily recognizable as belonging to the *Alternaria* genus with relatively small and short conidia on branching chains. Similar descriptions were given for several species of *Alternaria* on V8 agar medium by Simmons and Roberts (1993) and Simmons (1999, 2004).

Conidia of *Alternaria* sp. isolate Mlb684 are short ellipsoid to oval, tapering in the lower half into a narrow tail extension. The upper part which was materialized by a very short beak well rounded ending abruptly appears allowing the formation of new spores, thus furnishing evidence of catenulation.

Primary conidiophores of *Alternaria* sp. isolate Mlb684 arise directly from hyphae at the V-8 agar surface; they can be simple or branched. Myceliums are septate and the conidia (see illustrations in Fig. 2A, B) are variable in size and shape, but most often short and ellipsoid to oval, tapering in the lower half. Sometimes a narrow tail extension is visible. The upper part bears a beak, but it is very short and rounded; catenulation is frequent. To our knowledge the sporulation pattern observed here, characterised by an unusually high percentage of relatively small conidia produced in non-disjunct series, and has not been observed previously in any *Alternaria* species (Fig.2C). The spores are often well formed, with septa. Most of them have a smooth wall like those of *A. sesame*, *A. sesamicola*, and *A. simsimi* described by Simmons (2004). In cultures on potato-carrot agar, conidia E.G.S. 9-28 (-32) x 12-15µm in size, with transverse septa and at least one longitudinal septum, were observed (data not shown).

Figure 2. *Alternaria jacinthicola*. Septa mycelia (A) Conidiophore (B) and Arrangement of conidia (C) and ex representatives of isolate Mlb684; from development of foliar disease and colony developed on V8 agar (100x magnification).

IV.3.3. Taxonomy

Comparisons of DNA sequences, elongation factor 1-alpha gene, calmodulin gene and actin gene and morphology of isolate Mlb684 from Mali in the *Alternaria* genus revealed that the fungus from Mali represents a previously undescribed species in the genus. A new species are described as follows.

Alternaria jacinthicola Dagno & MH. Jijakli sp. nov.

Ex cultura in agaro V-8 descripta. Conidiophora abundantia, congesta ad ca. 70 x̣ 2–4 μm, ad apicem geniculata, conidiogena. Conidia catenulata, filamentoso. Rostrum brevissimum rotundata saepe corpore fusa ex conidium. Corpora conidiorum anguste ellipsoidea, ad 9-28 (-32) x̣ 12–15 μm; 3–7 constrictetransversaliter septata, 1(–2) longiseptata in segmentis 1–2 transversaliter; color modice brunneolus. Paries externus laevis. Habitatio typi in herba *Eichhornia crassipes*, Mali, (Dagno et al., 2011a).
Ex - Cult. Typ. BCCM^{TM}/MUCL = MUCL 53159.
Etymology- referring to the original host plant, water hyacinth.

Description and observations on the ex-type culture are based on the isolate Mlb684, which was derived from infected water hyacinth plant. The material was collected in 2006 in Mali. Colony growth on V-8 and PCA is rapid, completely covering individual sectors of 90 mm diameter Petri dish within 2 weeks. Sporulation is dense on V-8, only slightly less so on PCA. Concentric rings of sporulation are evident. The conidia are obclavate (shaped like a bowling pin) and form single file chains. The spores have 1 or 2 longitudinal and horizontal septae. Each conidium tapers into a narrow rounded protuberance. Conidium bodies reach a size range of 9-28 (-32) x̣ 12–15 μm, with 3-7 constricting transepta and 1 or 2 longiseptum in 1–2 transverse sections of narrow conidia.

IV.4. Discussions

Two species of *Alternaria* genus (*A. eichhorniae* and *A. alternata*) were previously described as pathogenic fungi on water hyacinth (Aneja et al., 1989; Nag Raj and Ponnappa, 1970). This study records the discovery of an unknown species in the *Alternaria* genus. Comparisons of DNA sequences, elongation factor 1-alpha gene, calmodulin and actin of isolate Mlb684 and all Alternaria DNA sequences and others DNA sequences existing in GenBank suggest that this fungus represents a new taxon, for which the name *A. jacinthicola* was provided.

To date, no further examination of molecular relationships among *Alternaria* sp. isolate Mlb684 and other genera has been explored. Moreover, no analysis of this isolate Mlb684 phylogeny has ever been conducted. This work provides the first systematic examination of isolate Mlb684 as they relate to the hypothesized related taxa of *Alternaria* (Simmons 1992).

Moreover, it should be noted that some *Alternaria* species were already shown to infect water hyacinth and have been assessed as potential biocontrol agents against this weed. These are *A. eichhorniae* in Egypt (Shabana, 2005) and *A. alternata* in India (El-Morsy et al., 2006; Babu et al., 2002) which caused severe disease on the plant in greenhouse test conditions. Genetic comparisons of with *Alternaria* sp. isolate M1b684 with DNA sequences from these *Alternaria* isolates had not been possible in absence of genetic characterization of

these organisms, at the exception of one sequence of *A. eichhorniae*. Additionally, isolate of *A. eichhorniae* in GenBank is not the one originally described by Shabana (1995) but by Nag Raj and Ponnappa on water hyacinth in India (Nag Raj and Ponnappa, 1970).

The objective of this phylogenetic study is to examine the relationships among *Alternaria* sp. isolate Mlb684 and the *Alternaria* genera and the closest species or isolates based on mitochondrial rDNA sequences for which sequences were available in Genbank.

Because ITS sequence of *Alternaria* sp. isolate Mlb684 was then 99% identical to those of 93 other isolates making it not pertinent to build a molecular phylogeny. In addition, the 18S rDNA gene sequence of *Alternaria* sp. isolate M1b684 showed also than a 98 to 99% identity with those of 72 other *Alternaria* or unknown cultured fungi isolates making so it not pertinent to build his molecular phylogeny.

For morphological description, the taxon of isolate Mlb684 does not appear to be one that is identifiable with those currently recognized by E.G.S. as described in the literature (Simmons, 2004; Simmons, 1999; Simmons and Roberts, 1993). Genetic results confirmed that the fungal isolate M1b684 belonged to the genus *Alternaria* and was distinct from any *Alternaria* species and isolates which had been previously characterised.

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CHAPTER V

PRODUCTION AND OIL-EMULSION FORMULATION OF *CADOPHORA MALORUM* AND *ALTERNARIA JACINTHICOLA*, TWO BIOCONTROL AGENTS AGAINST WATER HYACINTH (*EICHHORNIA CRASSIPES*)

PRODUCTION AND OIL-EMULSION FORMULATION OF *CADOPHORA MALORUM* AND *ALTERNARIA JACINTHICOLA*, TWO BIOCONTROL AGENTS AGAINST WATER HYACINTH (*EICHHORNIA CRASSIPES*)

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Abstract

Cadophora malorum isolate Mln715 and *Alternaria jacinthicola* strain MUCL 53159 are under development as biocontrol agents against water hyacinth (*Eichhornia crassipes*) in Mali. Production of spores of these agents on locally available substrates (water hyacinth, powdered paddy rice chaff, wheat semolina) was assessed with a view to mass production. The *C. malorum* isolate sporulated best on water hyacinth (4.08×10^7 spores ml^{-1}), followed by wheat (1.06×10^7 spores ml^{-1}), whereas *A. jacinthicola* produced more spores on paddy rice chaff and wheat (0.24×10^7 spores ml^{-1}). The severity of the damage caused by each pathogen was evaluated in the greenhouse and in the field. Under both greenhouse and field conditions, the biocontrol efficacy of the fungal isolates was improved with (unrefined) *Carapa procera* (L.) oil or (refined) palm oil, supplemented with soybean lecithin and Tween 20. When such a formulation was used, the incubation time was 4 to 5 days in the greenhouse and 7 to 9 days on the field, and the damage severity (DS) recorded 6 weeks after treatment varied from 87.02 to 93.13% in the greenhouse and from 59.11 to 63.00% in the field. For unformulated *C. malorum* and *A. jacinthicola* respectively, the incubation times were longer and the DS values were only 22.11 and 29.05% in the greenhouse and 12.05 and 15.15% on the field. Our results highlight good substrates for mass production of these mycoherbicides and demonstrate the ability of vegetable oil formulations to improve their efficacy.

Keywords: water hyacinth, formulation, biocontrol, *Cadophora malorum*, *Alternaria jacinthicola*.

V.1. Introduction

Water wyacinth [(*Eichhornia crassipes* (Mart.) Solms-Laubach] originating from the Amazon Basin in South America is a free-floating aquatic weed that is found worldwide. *Eichhornia crassipes* belonging to the family *Pontederiaceae* is considered the world's worst aquatic weed (Lata and Dubey, 2010). It causes global annual losses in excess of US$ 100 million for hydro-electricity plants, irrigation schemes, fisheries, riparian communities, and activities relying on water transit (Adebayo and Uyi, 2010**;** Gopal, 1987). Since 1980, rice and irrigated orchards have been the most important sources of food and income for farmers in Mali, especially in the Koulikoro, Segou, and Niono regions along the Niger River (Dagno, 2006). Many diseases, insects, and weeds, however, limit the stability of rice and fruit production. In particular, infestation of the Niger River by water hyacinth has increased drastically. This aquatic weed decreases the water flow by clogging dams and irrigation amenagment.

Herbicides (Paraquat, Diquat, Glyphosate, Amitrole, 2,4-D acid or amine) have been widely used to manage water hyacinth in water bodies (Land Protection, 2001), but their residual toxicity and deleterious effects have prompted the search for eco-friendly alternatives for controlling the weed (Gupta et al., 2002). An alternative envisaged is biological weed control based on the use of natural microorganisms. The classical approach involving the use of exotic plant pathogens was developed in the early 1970s (Watson, 1991). *Puccinia chondrilla*, fort example, has been used effectively to control skeleton weed (*Chondrilla juncea*) in Australia and the U.S.A (Yang et al., 2000). Another approach is to use an endemic (native) pathogen to control its host weed, by delivering a massive dose of the pathogen during susceptible stages of weed growth (Charudattan 1991). Over the last decade, much research has been devoted worldwide to the development of new mycoherbicides. In particular, the following species have undergone extensive testing for commercial development: *Colletotrichum gloeosporioides* f. sp. *aeschynomene* for northern jointvetch (Bowers, 1986), *C. coccodes* for velvet leaf (Wymore et al., 1988), and *Alternaria eichhorniae*, (Shabana, 2005), *A. alternata* (Babu et al., 2003), *Sclerotinia sclerotiorum* (de Jong et al., 2003), and *Cercospora rodmanii* (Charudattan, 1996) for water hyacinth.

In 2006-2007, our team conducted a survey of fungal species present on water hyacinth in Mali (Dagno et al., submitted). Two isolates emerged as promising potential biocontrol agents against this weed: *Cadophora malorum* (Syn. *Phialophora malorum*, *Sporotrichum malorum*) isolate Mln715 and *Alternaria* sp isolate Mlb684, now called strain MUCL 53159, representing a new species called *Alternaria jacinthicola* (Dagno et al., also submitted). In keeping with the conclusion of Boyette et al. (2007) that water activity and temperature are the two most important environmental factors influencing mycoherbicide efficacy, the germination potential and mycelial growth of these two strains show a clear temperature optimum (near 25°C) and are negatively affected by low water activity (Dagno et al. 2010). Such influences make it important to consider with care the the formulation of biocontrol agents, recognised as an important determinant of their efficacy (Greaves et al. 1998) (Greaves et al. 1998). Furthermore, having a cheap and locally available substrate on which to mass-produce inocula can also be important (Siddiqui et al., 2008).

The objective of the present work was to determine, under greenhouse and field conditions, the effects of oil-water emulsions (prepared with unrefined *Carapa procera* (L) oil or refined palm oil and amended with soybean lecithin and Tween 20) on the germination of *C. malorum* (isolate Mln715) and *A. jacinthicola* (strain MUCL 53159) spores and on the

severity of the damage they cause to water hyacinth. We have additionally tested three locally available plant materials as substrates for economically viable mass production of *C. malorum* and *A. jacinthicola* strain MUCL 53159.

V.2. Materials and Methods

V.2.1. Fungal and plant preparation

Cadophora malorum isolate Mln715 is stored at the Plant Pathology Unit, Gembloux Agro-Bio Tech - University of Liege, Belgium (at -70°C, in tubes containing 25% glycerol). *Alternaria jacinthicola* strain MUCL 53159 is stored in the Industrial Fungus & Yeast Collection (BCCM™/MUCL) in Belgium. The initial conidial inocula used in the present experiments were taken from Petri-dish cultures on Potato Dextrose Agar (PDA, Merck, Darmstadt, Germany), preserved at 4°C for no more than 6 months, and then subcultured at 25°C on different culture media before use. Healthy water hyacinth plants were collected and sampled from a naturally infested area of the Niger River in Mali and grown in a sterilized greenhouse.

V.2.2. Evaluation of potential substrates for mass production of fungal strains

Commercial (wheat) semolina, powdered glumes of paddy rice, and ground stems and leaves of water hyacinth were tested as potential substrates for mass production of fungal inocula. The substrates were sterilized at 120°C for 15 min. The water activity of all media was measured with an AquaLab 3TE (Decagon Device, Inc. 2365 NE Hopkins Court Pullman, WA 99163 USA). It was adjusted by adding distilled water to obtain 0.990 at 25°C. Sterile glass bottles with cotton stoppers were used, each containing 1000 g substrate. Their content was sprayed with 3, 0.3, or 0.5 l suspension containing 1 x 10^5 spores ml^{-1} of fungal isolate. After four weeks, the bottles were opened and watered with 50 ml sterile distilled water containing 5% Tween 20. To facilitate the detachment of conidia, the mixture was homogenized on a rotary shaker at 120 rpm for 15 min. To remove the mycelial mass and residual substrate, the suspension was filtered through cheesecloth. The concentration of the conidial suspension was determined with a Burker cell. This experiment was conducted twice with three replicates.

V.2.3. Conidia viability

The viability of the *Cadophora malorum* and *Alternaria jacinthicola* conidia produced on different substrates was evaluated on water hyacinth under controlled conditions. Actively growing plants at the 3- to 5-leaf stage were inoculated with 20 ml fungal strain in aqueous suspension at 1 x 10^5 spores ml^{-1}. Treated plants were placed in a greenhouse at 55 ± 5% relative humidity and 25 ºC with a 16-h photoperiod. Conidia were harvested from inoculated leaves and rinsed with sterile distilled water. Finally, 100 conidia were examined under 40x and 100x magnification (Egley et al.1995) for germination.

V.2.4. Evaluation of the efficacy of vegetable oil emulsion formulations under greenhouse conditions

Vegetable oil emulsions were prepared with 35% v:v refined palm oil or unrefined *Carapa procera* (L) oil amended with 15% soybean lecithin (used as an emulsifying agent) and 5% Tween 20 (used as a formulation adjuvant)(all concentrations mentioned are final concentrations). On the one hand, Tween 20 was diluted in sterile distilled water containing the spores (at a concentration calculated to yield a concentration of 5 x 10^6 spores ml^{-1} in the final oil-water emulsion). On the other hand, preheated soybean lecithin was added to the

vegetable oil phase and the resulting mixture was homogenised with a blender. The oil and aqueous phases were then combined and vigorously homogenized with an electric mixer.

Each oil-emulsion formulation was sprayed onto hyacinth plants at the 3- to 5-leaf stage. Three types of controls were also included: "Control I" plants were sprayed with unformulated fungus (50 ml of the aqueous phase described above, containing 5 x 10^6 spores ml^{-1}), "Control II" plants with sterile distilled water without fungus, and "Control III" plants with vegetable oil only. Treated plants were incubated under the same conditions as described above. Three replicates were prepared for each treatment and arranged in a complete randomized design. After six weeks, the plants were rated for disease symptoms including leaf spots, leaf lesions, and leaf death. The impact of the pathogens was determined by counting the number of leaf spots and leaf lesions per leaf and by assessing the damage severity (DS). The DS was derived according to Freeman and Charudattan (1984), from ratings on a scale of 0 to 9, where 0 = healthy, and 9 = 100% damage. Scores for individual leaves were summed and averaged, and the mean score converted to a percentage for a whole plant. The experiment was repeated three times.

V.2.5. Field trials

In 2009, two independent field trials were conducted to assess the biocontrol efficacy of our two fungal pathogens against this weed. These trials were conducted in two infested areas on the River Niger in the District of Bamako. Nocturnal temperature (recorded at midnight), diurnal temperature (recorded at 2 PM), and relative humidity (RH) were recorded during the experiment. Each treatment (unformulated or formulated pathogen, oil alone, distilled water) was sprayed over a 2m x 2m area (1000 ml m^{-2}). Treatments were arranged in a randomized complete block design with three replicates. The incubation period was determined and leaf blight severity was assessed on the whole water hyacinth leaves of each treatment area 6 weeks after treatment (as described above).

V.2.6. Data analysis

Analysis of variance (ANOVA) was applied to the production and damage severity data. The software package used was SAS 9.1 (SAS Institute, Cary, NC, USA). When effects proved significant, Duncan's multiple range test was employed for mean separation at the $P<0.05$ level.

V.3. Results

V.3.1. Conidial yield and viability

Figure 1 shows the sporulation rates of fungal isolates on different plant substrates. The differences shown varied from significant ($P < 0.05$) to highly significant ($P < 0.001$). *Alternaria jacinthicola* sporulated somewhat better on both paddy rice and wheat than on water hyacinth (Fig. 1A), whereas *C. malorum* sporulated best on water hyacinth, followed by wheat (Fig. 1B). The conidial yield of *A. jacinthicola* was consistently lower than that of *C. malorum* (respective maximum yields recorded: 0.24×10^7 vs.4.08×10^7 spores ml^{-1}).

The viability of the *C. malorum* and *A. jacinthicola* conidia produced on these plant substrates was evaluated on water hyacinth leaves. Whatever the pathogen, germ tubes were clearly visible on leaves (at 100x magnification) 4 hours after inoculation. The mycelium was found to colonize the leaf surface and branches before entering through stomata or intercellular spaces and colonizing the whole leaf 8 hours after inoculation (data not shown).

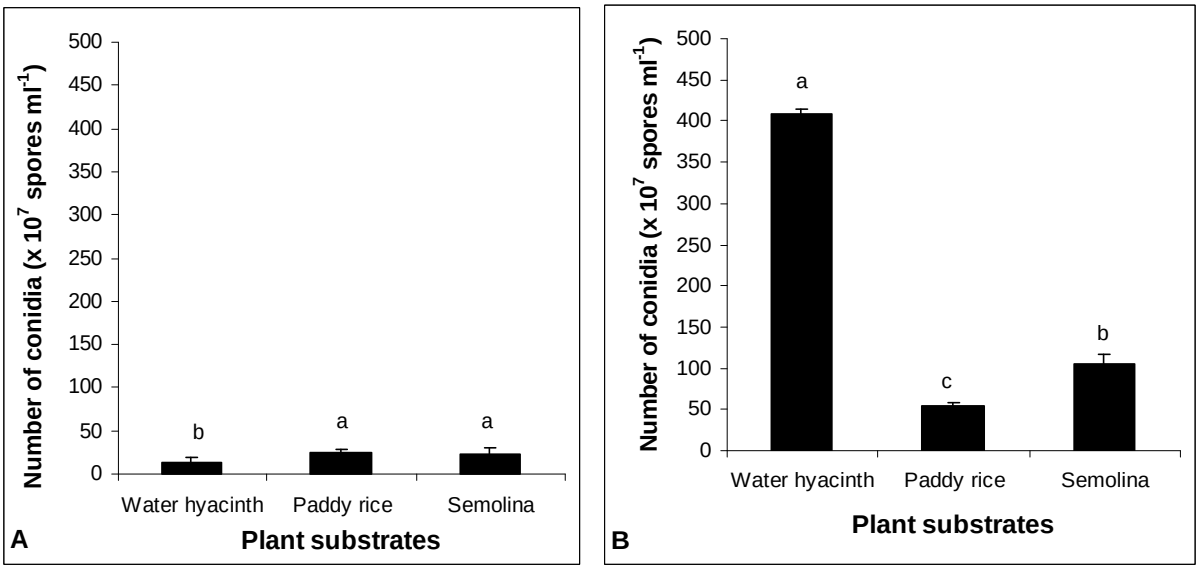

Figure 1. Production of biocontrol agents on plant substrates 4 weeks after incubation. (A) *Alternaria jacinthicola* (strain MUCL 53159) ; (B) *Cadophora malorum* (isolate Mln715). Treatments with the same letters are not significantly different according to Duncan tests ($P < 0.05$). Bars represent the standard error of the means.

V.3.2. Evaluation of vegetable oil emulsion efficacy under greenhouse conditions

A preliminary small-scale greenhouse trial was conducted at the Plant Pathology Unit, Gembloux Agro-Bio Tech to evaluate the biocontrol efficacy of both fungal isolates against water hyacinth. The incubation or latency period (time elapsed between exposure to a pathogen and the appearance of the first symptoms); the number of diseased leaves, and the leaf area covered by disease were estimated. In this experiment, all oil-emulsion-treated samples (including the pathogen-free controls) showed slight leaf necrosis from day 1 post-inoculation onward, but the onset of symptoms due to either pathogen was nevertheless clearly distinguishable. With both fungal species, the latency period was 4 days for the pathogen formulated in palm oil and 5 days for the pathogen formulated in *Carapa procera* (L) oil. Plants pulverized with unformulated *C. malorum* or *A. jacinthicola* (Control I) showed

a 9-day latency.

The number of foliar lesions counted after 6 weeks was highest (83 for *A. jacinthicola* and 110 for *C. malorum*) when the pathogen tested was formulated in palm oil. It was lowest when the tested fungus was unformulated. Table 1 shows the damage severity percentages recorded 6 weeks after treatment. At this time, in the plants treated with refined palm oil, the DS reached 90.35% (*A. jacinthicola*) to 93.13% (*C. malorum*). With unrefined *Carapa procera* (L.) oil, the DS was 87.05% for both fungal isolates. The unformulated pathogens caused much lesser damage: 22.11% for *A. jacinthicola* and 29.05% for *C. malorum*. The DS due to phytotoxicity of the vegetable oil used alone was 6.10% for *Carapa procera* (L.) vs. 5.25% for palm oil. The controls treated with distilled water alone developed no symptoms at all. It is noteworthy that the plants treated with oil-formulated pathogens developed buds but no daughter plants over the 6-week period, whereas Controls I, II, and III produced respectively 5, 16, and 12 daughter plants. Figure 2 shows the severest symptoms developed on water hyacinth treated with biocontrol agents.

Table 1. Efficacy of variously formulated biocontrol agents against water hyacinth under greenhouse conditions (relative humidity = 55 ± 5; temperature = 25°C; 16-hour photoperiod). DS values were recorded 6 weeks after treatment.

Treatment	**DS (%)**
Trt 1	93.13±0.42
Trt 2	87.02±1.02
Trt 3	90.35±0.50
Trt 4	87.05±1.09
Control I1	22.11±1.32
Control I2	29.05±1.52
Control II	0.00±0.00
Control III1	5.25±0.00
Control III2	6.10±0.00

DS: Damage severity, Trt 1: *Cadophora malorum* + palm oil, Trt 2: *Cadophora malorum* + *Carapa procera* (L) oil, Trt 3: *Alternaria jacinthicola* + palm oil, Trt 4: *Alternaria jacinthicola* + *Carapa procera* oil, Control I1: *Cadophora malorum* unformulated, Control I2: *Alternaria jacinthicola* unformulated, Control II: distilled water, Control III1: palm oil, Control III2: *Carapa procera* (L) oil.

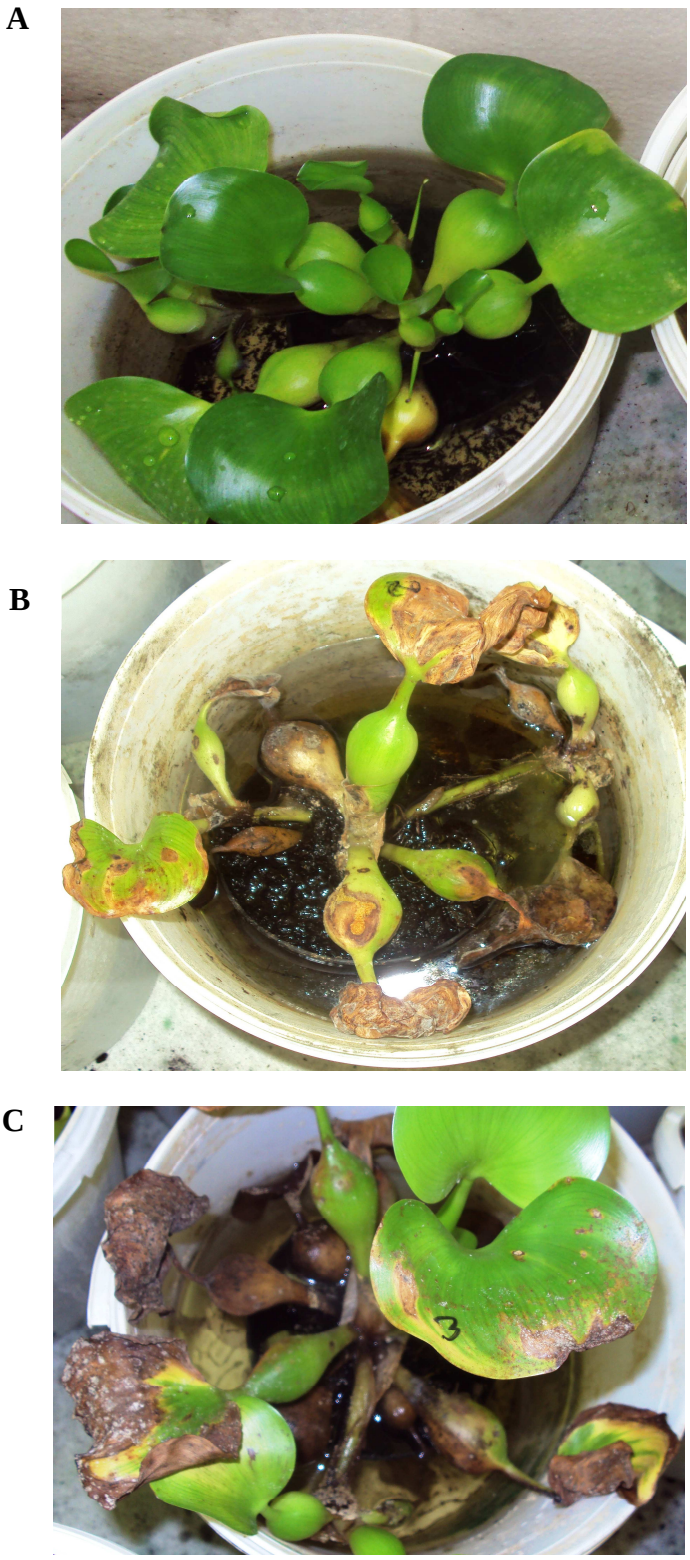

Figure 2. Healthy water hyacinth plant (A); leaf blight symptoms caused by *Cadophora malorum* (isolate Mln715) (B) and by *Alternaria jacinthicola* (strain MUCL 53159) (C) 6 weeks after inoculation of greenhouse-grown water hyacinth plants. The treatments B and C are with palm oil.

V.3.3. Field trials

Field trials were conducted in naturally infested parts of the Niger River in the District of Bamako in Mali. In these trials, the incubation period for oil-formulated *C. malorum* was 7 (palm oil) or 8 days (*Carapa procera* oil), as opposed to 12 days in the case of the unformulated pathogen. The incubation period for *A. jacinthicola* was 9 days (*Carapa procera* oil), 8 days (palm oil), or 12 days (unformulated). Whatever the oil, the oil-formulated fungi showed very similar efficacy (DS = 59.11 to 63.00%), the only significant difference being a slightly lower DS for *C. malorum* in *Carapa procera* oil (Table 2).

Plants treated with oil alone again showed slight necrosis from day 1 post-pulverisation onward, and in the distilled water controls, slight symptoms appeared after 13 days (DS = 5.20% after 6 weeks). Over the test period (February and March 2009), the average temperature recorded at 2:00 PM was 35°C and the average temperature recorded.

Table 2. Efficacy of different formulations of *Cadophora malorum* (isolate Mln715) and *Alternaria jacinthicola* (strain MUCL 53159) against water hyacinth in the field (DS recorded 6 weeks after pulverization).

Treatment	**DS (%)**
Trt 1	63.00±3.60
Trt 2	59.11±2.75
Trt 3	63.02±3.10
Trt 4	62.21±2.32
Control I1	12.05±5.20
Control I2	15.15±4.80
Control II	5.20±5.30
Control III1	9.20±3.85
Control III2	10.18±3.25

DS: Damage severity, Trt 1: *Cadophora malorum* + palm oil, Trt 2: *Cadophora malorum* + *Carapa procera* (L) oil, Trt 3: *Alternaria jacinthicola* + palm oil, Trt 4: *Alternaria jacinthicola* + *Carapa procera* oil, Control I1: *Cadophora malorum* unformulated, Control I2: *Alternaria jacinthicola* unformulated, Control II: distilled water, Control III1: palm oil, Control III2: *Carapa procera* (L) oil.

V.4. Discussion

Despite efforts to control it, water hyacinth continues to cause serious economic, social and environmental problems in Mali (Dagno 2006). In recent years, attention has focused on biological control as a cost-effective, eco-friendly approach to controlling water hyacinth. Various authors report high levels of biological control achieved with fungal pathogens (El-Morsy, 2006; Shabana et al., 2005; Vincent, 2001).

Here we have focused on two fungal pathogens isolated from diseased water hyacinth plants in Mali, with a view to developing them as effective biocontrol agents. We have notably considered the problem of local mass production. Several plant substrates such as wheat straw; chickpea flour; rice straw, sorghum, and rice have been used to mass produce BCAs (Siddiqui et al., 2008; Abbas et al., 2000), but the use of water hyacinth and paddy rice chaff as substrates for mass production of *Cadophora malorum* and *Alternaria* species has not been reported previously. In our tests, the former substrate proved best for producing *C. malorum* and the latter for producing *A. jacinthicola*. These substrates should be obtainable locally at low cost, as water hyacinth is an abundant weed and rice chaff, an industrial waste. It should also be relatively simple to use them in developing countries where water hyacinth is considered an important threat and where fermentation systems for inoculum production are lacking. In our case, all three tested substrates yielded spores that germinated well on water hyacinth.

We have also considered the formulation of these fungi. On the one hand, an adequate formulation can contribute to a uniform distribution of inoculum on the plant surface, essential to creating epidemics (Boyette et al., 2007). On the other hand, its use may help to solve a major problem inherent in using leaf fungi for weed control: the fact that such fungi require optimal relative humidity for germination, in order to infect the weed (Shabana, 2005). In relation to this problem, El-Morsy (2006) reports that oil emulsion formulations may reduce dew requirements in field application.

According to Shabana et al. (2005), the effect of a mycoherbicide on a weed can be determined on the basis of its ability to reduce development of the host plant, fewer living leaves and more dead leaves on individual plants, and no production of new clones. Here we have tested formulations of *C. malorum* or *A. jacinthicola* in 35% v/v emulsions of refined palm oil or unrefined *Carapa procera* (L) oil (oils readily available and easy to use), comparing their performance with that of the unformulated fungi. We demonstrate a 3- to 4-fold increased DS under greenhouse conditions (at low RH) and a 4- to 5-fold increased DS under field conditions, when such formulations are used against water hyacinth. We also show that these formulations decrease the disease incubation time and significantly inhibit daughter plant formation. The increased efficacy of oil-formulated BCAs may be due to decreased water loss by the weed through evaporation and transpiration (Shabana, 1997; Pieterse et al., 1990).

Despite the improvements recorded with our oil formulations, our *C. malorum* and *A. jacinthicola* strains proved less effective against water hyacinth on the field than in the greenhouse. Similar observations have been made with formulations of *B. bassiana* and *M. anisopliae* (Alves et al., 1998). Such results may be due to factors such as intense solar irradiation (decreasing BCA viability in the field), major variations in temperature and RH between night and noon (in our case, 15°C and 55% RH at midnight vs. 35°C and 85% RH at 2:00 PM), or other microenvironmental factors.

There is thus room for further improving the biocontrol efficacy of our strains. A simple solution might be to increase the concentration of the inoculum, so as to offset any viability-diminishing effects of environmental factors. Boyette et al. (2007) achieved higher efficacy of *C. truncatum* against hemp sesbania in field trials with 1 x 10^7 spore ml^{-1}. Another approach might be to change the timing of *C. malorum* or *A. jacinthicola* application. Our field assays were performed from February to March, a dry season in Mali. The period from June to October, characterised by rainfall, slighter temperature variations, and good growth of water hyacinth, might be better. Further improvements to the formulation might also be considered. Incorporation of lignin or other UV-protectants into the formulated product, in combination with skimmed milk or another carbon source, may protect a fungal isolate against desiccation and harmful effects of UV light and thus prolong its persistence. It has been demonstrated, for instance, that formulating *B. bassiana* spores with lignin can significantly increase their survival under solar radiation (Leland et al., 2005).

Our results show that the biocontrol efficacy of *Cadophora malorum* isolate Mln715 and *Alternaria jacinthicola* strain MUCL 53159 can be improved by formulating these BCAs in an emulsion of refined palm oil or unrefined *Carapa procera* (L.) oil emulsion. These oils offer an effective, easy-to-use option as a formula for the mycoherbicide. The efficacy of these formulations might be enhanced by adding lignin and/or a carbon source such as molasses. Increasing the concentration of these BCAs might also allow better control of water hyacinth than achieved here.

V.5. Conclusion

Our results show that the biocontrol efficacy of *Cadophora malorum* isolate Mln715 and *Alternaria jacinthicola* strain MUCL 53159 can be improved by formulating these BCAs in an emulsion of refined palm oil or unrefined *Carapa procera* (L) oil emulsion. These oils offer an effective, easy-to-use option as a formula for the mycoherbicide. The efficacy of these formulations might be enhanced by adding lignin and/or a carbon source such as molasses. Increasing the concentration of these BCAs might also allow better control of water hyacinth than achieved here.

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CHAPTER VI

GENERAL DISCUSSIONS

DISCUSSIONS

Since 1990, water hyacinth continues to pose serious economic, social and environmental problems in Mali (Dagno, 2006). In recent years, attention has been given to biological control as a cost-effective and environmentally safe method for managing the water hyacinth infestation. Fungal pathogens have been shown to be the best effective biocontrol agents (El-Morsy, 2006; Shabana et al. 2005; Vincent 2001) (Chapter I). Therefore, our works presented here constitute an original investigation on screening and development of potential biocontrol agents for the control of water hyacinth infestations in Mali.

To the best of our knowledge, this is the first time that such extensive survey of phytopathogenic fungal isolates on water hyacinth has been conducted. Until there, none of fungal isolates has been reported on this weed in Mali (Chapter II). Among our fungal isolates, *Alternaria* and *Fusarium* are ubiquitous fungal genera and have been isolated from almost all inhabitats infested with water hyacinth worldwide (Evan et al., 2001; Martinez et al., 2001). Both genera include many species or strains that may be pathogenic towards several crops (Babu et al., 2003; EL-Morsy, 2000). They also include some strains that appear specifically pathogenic against water hyacinth. Fungal isolates such as *A. eichhorniae*, *A. alternata*, and *F. pallidoroseum* have been reported as promising biocontrol agents against water hyacinth in Egypt and India (Shabana, 1997; El-Morsy, 2004; Naseema et al., 2004). We are the first to report the presence of genus *Cadophora* on water hyacinth in worlwide. This genus, however, is recognized as pathogenic for many crops like corn, rice, apple, and pear (Frisullollo, 2002; Benbow et al., 2002). Under greenhouse trials, our 3 most effective potential fungal isolates have caused 70% (isolates Mln799, Mln715) or 71% (isolate Mlb684) of foliar lesions on water hyacinth. The present results highlight that *Fusarium* sp. isolate Mln799, *Cadophora* sp. isolates Mln715, and *Alternaria* sp. isolate Mlb684 could be considered as potential bioherbicides (Chapter II). These isolates were identified as *Gibberella sacchari*, *Cadophora malorum*, and *Alternaria* sp. respectively by Industrial Fungal & Yeast collection (BCCM/MUCL) - Belgium.

The effectiveness of pathogenic fungal isolates tended to be greater with lesser variation in greenhouse conditions than in field trials as previously reported by Boyette et al. (2007). It has been shown that environmental factors such as temperature, water activity, RH and UV radiations influenced the efficacy of weed biocontrol agents under field conditions (Charudattan, 2005; Babu et al., 2003).

Until now, no reports in the literature have highlighted the effects of these factors on spores germination and mycelial growth of the above-mentioned strains. Therefore, our Chapter III investigated the germination and mycelial growth rates of the three fungal pathogens, *F. sacchari* (isolate Mln799), *C. malorum* (isolate Mln715), and *Alternaria* sp. (isolate Mlb684) according to the studied factors (temperature and water activity). Using glycerol-supplemented media under *in vitro* conditions, the combined effects of temperature and water activity on spores germination and mycelial growth were assessed.

The water activity emerged as a crucial determinant factor for germination of all fungal isolates. Their germination being fastest at $a_w = 0.96$ (reaching 100% at 25°C within 24 h) and slowest at $a_w = 0.88$. This result is in agreement with those previously reported on *Monilinia fructigena* and *M. fructicola*, two apple brown rot fungi (Xu et al., 2001; Tamm et al., 1993). Regarding the growth rate, our results show that temperature and a_w are key determinant factors for the growth rate of pathogenic fungal isolates. Again, water activity

emerges as the factor having the greatest influence. Whatever the temperature, no growth was observed at a a_w of 0.88 for both isolates Mlb684 and Mln799 while there was a low growth for the isolate Mln715 at 15 and 25°C. These results are in agreement with previous findings indicating that the amount of available water in the substrate and the surrounding environment is very important for fungal growth (Lahlali et al., 2008). *F. sacchari* (isolate Mln799) showed a higher tolerance to extreme temperature, whereas *C. malorum* (isolate Mln715) was more tolerant to water stress. Lasram et al. (2010) reported that the optimal growth of *A. carbonarius* was observed at temperatures ranging from 25 to 30°C and for $a_w = 0.99$. They mentioned that this fungus grows poorly at 15°C and at $a_w \leq 0.90$. Romero et al. (2010) likewise reported slow growth of *A. carbonarius* at low a_w and temperature (0.83 and 15°C). Selected models predict fairly accurately the growth rates of these three strains. At $a_w = 0.88$, for instance, the *C. malorum* (isolate Mln715) model correctly predicts slight growth at 15 and 25°C but none at 35°C. Howver the growth models of *F. sacchari* (isolate Mln799) and *Alternaria* sp. (isolate Mlb684) correctly predict no growth of either strain at 15 or 35°C. It is crucial to emphasize that our glycerol models for *F. sacchari* (isolate Mln799), *C. malorum* (isolate Mlb684), and *Alternaria* sp. (isolate Mlb684) are based on data obtained under *in vitro* conditions.

Our models might overestimate growth under natural conditions, because our isolates were grown on a nutrient-rich artificial medium under good light conditions. Furthermore, environmental factors other than those studied here may be involved, such as relative humidity, UV, pH, and interactions with organisms of the microflora present on the leaf surface of water hyacinth.

In addition, we have presented morphological and genetic evidence that *Alternaria* sp. isolate Mlb684 represents a new, previously unreported species within the genus *Alternaria.* The isolate was named "*Alternaria jacinthicola* Dagno & M.H. Jijakli" and we have deposited in GenBank the partial gene sequences on which our molecular analyses are based. These notably include the first GenBank entry of an *Alternaria* calmodulin gene sequence (Chapter IV).

Greaves et al. (1998) reported that formulation is the good way to increase both efficiency of application and biocontrol efficacy. Oil emulsion formulations, in particular, may reduce dew requirements in field application (El-Morsy, 2006). To our knowledge, this study is the first regarding the development of mass production methods of *Cadophora malorum* (isolate Mln715) and *Alternaria* sp. (isolate Mlb684) on vegetable substrates (Chapter V). Fungal inoculum produced on paddy rice and water hyacinth substrates showed to be more efficacious than wheat semolina for water hyacinth plant control.

The spraying of water hyacinth plants in the greenhouse with *C. malorum* (isolate Mln715) and *Alternaria jacinthicola* (isolate Mlb684) without formulation have not provided an average efficiency, 22.11 and 23.05% DS respectively. In addition, *C. malorum* and *A. jacinthicola* were formulated with unrefined oil based on *Carapa procera* (L) and refined palm oils to improve significally their efficacy as mycoherbicide (93.13 and 90.35% DS with *C. malorum* and *A. jacinthicola* respectively in palm oil).
These results have confirmed those obtained in a greenhouse study on the impact of environmental factors on the development of two biological control agents. They need high humidity to germinate and penetrate the plant cell host. This constraint is circumvented by the development of pathogens in vegetable oils.

In field application, effective control of *C. malorum* and *A. jacinthicola* (63.00 and 63.02% DS, respectively) formulated in palm oil has substantially fallen. Field results showed that *C. malorum* and *A. jacinthicola*, applied in combination with vegetable oil, failed to infect and produce the disease whereas they did when they were applied in a greenhouse. Alves et al. (1998) and Burges (1998) reported similar results on formulation of *B. bassiana* and *M. anisopliae* in field application. This result could be explained by higher significant variation in temperature between night and noon. The air temperature was 15 and 35°C at 23 and 14 hours respectively. Field assays were performed on period extend from February to March which corresponds to a dry season in Mali. During this period, extreme temperature differences were observed between night and days (10-15 and 35-45°C respectively). This means that improving the formulation of *C. malorum* and *A. jacinthicola* poses an additional challenge to obtain high efficacy in field application.

Whatever these biocontrol agents, palm oil gave better protection against pathogens desiccation and produced a greater efficiency of water hyacinth control in greenhouse.

CHAPTER VII

GENERAL CONCLUSIONS AND PERSPECTIVES

GENERAL CONCLUSIONS

After twenty years of presence, *Eichhornia crassipes* has become the worst widespread aquatic weed in Mali. Its control is possible through physical, chemical or biological treatments. However, mechanical removal and herbicidal treatments have been found to be inadequate or too expensive notably in situations of large-scale proliferation of water hyacinth. Therefore, the main efforts should be focused on development of sustainable control methods based on biological approaches using plant pathogens.

In order to identify pathogens that can help controlling the weed we collected diseased water hyacinth plants in major areas of Mali in which its presence was considered as an agricultural and ecological problem. To date, we have surveyed and collected 1000 samples from the District of Bamako, Koulikoro and Segou regions. One hundred and sixteen fungal isolates have been identified on all samples of diseased plants. On the basis of morpho-biology and conidial arrangement, 7 genera have been identified. The conidial shape and structure allowed us to identify some of the fugal isolates to the species level. Two isolates (Mln715 and Mlb684) were distinguished as the most pathogenic during efficacy tests against the water hyacinth. They constitute effective biocontrol agents against water hyacinth. Among them, isolate Mlb684 has been first identified as an *Alternaria* sp. by the Industrial Fungal & Yeast collection (BCCM/MUCL) in Belgium and further confirmed by Dr. EG. Simmons (USA). Isolate Mln715 was identified as *Cadophora malorum*. This is the first report highlighting the genus *Cadophora* on water hyacinth.

Alternaria sp. isolate Mlb684 has been also genetically characterized by Professor F. Lefort in Switzerland and the strain has been clearly appeared as a novel species of *Alternaria* genus. Five target regions of the genome [ITS ribosomal RNA gene, 18S ribosomal RNA gene, elongation Factor-1 alpha (EF1a) gene, calmodulin gene and Actin gene] were sequenced and analyzed. The sequencing of calmodulin gene into *Alternaria* genus has been reported for the first time in this work. On the basis of morphological description and molecular characterization, a status of new *Alternaria* specie has been given to isolate Mlb84. Accordingly, this isolate was called "*Alternaria jacinthicola* Dagno & M.H. Jijakli". This strain has been deposited in the Industrial Fungal & Yeast Collection (BCCM/MUCL, Louvain-La-Neuve, Belgium) under accession number MUCL 53159.

Cadophora malorum and *Alternaria* sp. (isolate Mlb684) being sensitive to low water activity, research on formulation of *C. malorum* (isolate Mln715) and *A jacinthicola* (MUCL 53159) with vegetable oil emulsions showed promising results. Furthermore, vegetable oil [refined palm oil and unrefined *Carapa procera* (L)] could improve the efficacy of these biocontrol agents as mycoherbicide for water hyacinth. These results may support the mechanical removal of water hyacinth in Mali. As they constitute an important contribution to the integrated control of water hyacinth, our results will be of great importance for Mali and several other countries in West Africa where this weed represent a major environmental and economic problem.

PERSPECTIVES

Future studies will focus on the identification and biochemical analysis of the toxin(s) produced by the news species *A. jacinthicola.*

It is a challenge to develop an effective bioherbicide that is acceptable for use in practical water hyacinth management programs in Mali. From this work, further activities will plan to determine the optimum inoculums concentration and to asses the influence of UV radiation on *C. malorum* (isolate Mln715) and *A jacinthicola* (MUCL 53159). The physiological and ultrastructural host responses should be explored, to furnish background information and understood the host–pathogen-environment system. Formulations of biocontrol agents may be enhanced by adding lignin and some carbon sources.

Nevertheless, the mixture of biocontrol agents and chemical herbicides (applied at low doses) could be tested for manging water hyacinth infestation into integreated control program. We continue to develop an effective mycoherbicide based on these screnned fungal isolates for controlling water hyacinth in Mali and West Africa. Before makating, different studies sould be considered to boost the mode of action and adequate time of application.
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