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THE POTENTIAL OF AQUATIC PYTHIUM SPECIES FOR THE BIOLOGICAL CONTROL OF CLADOPHORA GLOMERATA IN IRRIGATION SCHEMES IN SOUTH AFRICA

Report to the Water Research Commission on the Project
"An investigation into the use of pathogenic organisms
for the biological control
of the troublesome alga Cladophora glomerata."

by

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Executive Summary

Cladophora glomerata ("Draadalg") is a worldwide nuisance alga of natural and man made water bodies. In the summer rainfall region of South Africa it causes blockages of structures such as weirs and grates in irrigation canals leading to water loss, a serious problem in a dry land.

Present control methods of *C. glomerata* in irrigation canals in South Africa include mechanical removal or dosing with copper sulphate in reaction to large blooms of the alga. Both are expensive options with high labour or material costs. Neither option leads to long term control. Because of these problems, two different biological control options are in the process of being investigated, namely the use of sterile triploid Grass Carp, and use of pathogens. The latter is reported on here.

A previous project surveyed for indigenous pathogens of *C. glomerata* in irrigation canals in the summer rainfall of South Africa. Two Chytridiomycete species were commonly observed. Also a number of *Pythium* species were commonly isolated from *C. glomerata* during this survey. At least three species of *Pythium* are known to be pathogenic to a number of freshwater green algae, especially *Spirogyra* species. The marine red alga *Porphyra* is also attacked by *Pythium* species, which can cause a serious disease during mariculture. As *Pythium* species are also well known pathogens of plants, including in hydroponic (soilless) plant growing systems, it was suggested that their potential as biological control agents needed to be investigated, and this project was therefore initiated to achieve this goal.

The aims of the project were:

- Investigate the efficacy of selected organisms to control Cladophora glomerata under laboratory and field conditions.
- Investigate the mass production, viable and practical formulations for application of, and the optimal dosage rates of the selected organisms.
- 3. Determine the host range of the selected organisms to ensure crop safety.

The first and third aim were achieved, however as no Pythium isolate proved to be highly pathogenic in laboratory conditions, testing under field conditions was not investigated. The second aim was therefore also not pursued.

Further studies on the effect of temperature, pH and copper on the growth, zoospore production and zoospore mobility of *Pythium* isolates was investigated. Lastly, preliminary studies on the use of *Acacia mearnsii* (black wattle) bark for control of algal growth were carried out. Factors influencing the integration of the use of *Pythium* with other existing or potential control methods were also investigated.

Conclusions reached were:

- 1. The Pythium isolates tested are adapted to the conditions from which they were isolated.
- Copper treatment at 0.5 mg/l Cu⁺ is unlikely to kill all the Pythium mycelium present in a system, but it will reduce the potential inoculum and in particular all zoospores will be eliminated.

- 3. The Pythium species tested proved to be incapable of penetrating healthy algal cells, and therefore can't be considered as pathogens with the potential to be developed as a bioherbicide. These fungi are therefore not the causal organism of diseased C. glomerata observed previously, and the use of Pythium species as biocontrol agents against C. glomerata will not be pursued further.
- A few isolates of Pythium proved to have a high potential pathogenicity against certain of the crop plants tested.
- Acacia mearnsii bark proved to be algicidal (at high concentations) or to inhibit growth (at low concentrations) of C. glomerata.
- Fungal pathogens other than Pythium are present in the irrigation schemes, and may yet prove to be valuable biocontrol agents against C. glomerata.

Integration with copper treatment would be problematical due to the general toxicity of the chemical to organisms. However the indirect use of pathogens present in the irrigation schemes, after treatments such as water level fluctuation or dosing with low concentrations of A. mearnsii bark, or possibly even low concentrations of copper (<0.2 mg/l), may still prove to be valuable within an integrated management programme.

Recommendations made were:

- The role of organisms (insects and fungi) in reducing algal biomass after a stress treatment such as fluctuating the water level or a low dose of an algicide needs to be determined.
- The short and long term effects of copper treatment on organisms capable of attacking Cladophora glomerata need to be assessed, and their recruitment rates after copper treatment determined.
- The potential of Acacia mearnsii bark to control algal blooms needs to be assessed, as well as possible toxicological effects.
- Further efforts to isolate the observed pathogen of Cladophora glomerata present in at least some irrigation schemes need to be carried out, and its potential as a biological control agent tested.

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Glossary of Terms Used and of Growth Media

BBE: Acacia meansii (blackwattle) bark extract.

stock solution of 403g dry weight of bark in 3l water, the extract was decanted after a
week. Used at various concentrations where 1% solution ≈ 1.4 g bark/l water.

CMA: cornmeal agar

 40g of coarsely crushed yellow maize seed boiled in /l distilled water for 1 hour, 15g agar added, autoclaved.

IPM: Integrated Pest Management

 pest control programmes integrating all means of control, with the purpose of reducing chemical usage whilst improving productivity.

NS: nutrient solution

 40mg MgSO₄.7H₂O, 20mg KCl, 40mg NaHCO₃, 100mg Ca(NO₃)₂.4H₂O, 10mg K₂HPO₄ // distilled water

1/2NS: half strength nutrient solution

50% NS stock solution + 50% tap water).

SEW: soil extract water

1g soil // distilled water, stirred then left to stand overnight (not autoclaved)

WA: water agar

· 15g agar /l distilled water, autoclaved

WA+L: water agar with leaves

 with autoclaved pieces of sorghum leaves placed onto hardened water agar, 6 leaf pieces per petri plate.

Chapter 1: Introduction

Cladophora glomerata ("Draadalg") is a world-wide nuisance alga of natural and man made water bodies. In the summer rainfall region of South Africa it causes blockages of structures such as weirs and grates in irrigation canals leading to water loss, a serious problem in a dry land (du Plessis & Davidson 1996). A recent report gives a general review of the literature on the biology of C. glomerata, and details of the biology and environmental tolerances of this alga in a South African irrigation scheme (Orange–Riet) (Joska et. al. 2000).

Present control methods of *C. glomerata* in irrigation canals in South Africa include mechanical removal or dosing with copper sulphate in reaction to large blooms of the alga (du Plessis & Davidson 1996). Both are expensive options with high labour or material costs. Neither option leads to long term control. Because of these problems, two different biological control options are in the process of being investigated, namely the use of sterile triploid Grass Carp, and use of pathogens.

Biological control of weeds is the use of living organisms to control unwanted plants. The advantages of biological control are 1) that it is an environmentally safe method of control, 2) that the chance of resistance developing in the weed is remote, 3) that the health risks to humans, domestic animals and crop plants are minimal, and 4) that it is relatively cost-effective. The disadvantage of biological control is that because it involves the interaction of different organisms and their environment, results are variable. A successful biological control programme means that the target weed's population is reduced but not eliminated, and management problems could still be encountered. Biological control of weeds is an established science throughout the world, with many important successes. Insects in particular have been utilised, but also mites, fungi, and bacteria. Fish have also been used against aquatic weeds.

Of all the various aquatic weeds, floating macrophytes and emergent macrophytes have received the most attention for biological control. In South Africa insects have been successfully used for the biological control of water fern (Salvinia molesta) (Cilliers 1991a), water lettuce (Pistia stratoides) (Cilliers 1991b), parrots feather (Myriophyllum aquaticum) (Cilliers 1999), and red water fern (Azolla filiculoides) (Hill 1999). The same, and additional, insect agents have been used successfully against these weeds elsewhere in the world (Julien & Griffiths 1998).

World-wide the most serious problem floating macrophyte weed, water hyacinth (Eichhornia crassipes), has been successfully controlled in many tropical areas (Julien & Griffiths 1998). In South Africa it has been successfully controlled by insects in certain, but not all, areas (Hill & Cilliers 1999). Factors that have limited the success of biocontrol include unfavourable environmental conditions for the insect biocontrol agents, periodic flooding with agents being swept away, and lack of integration of the agents with mechanical and chemical control operations.

The first submerged macrophyte to receive attention world-wide in a biological control programme using insects was Eurasian milfoil (*Myriophyllum spicatum*) in the USA with an indigenous moth (Batra 1977). Surveys for insect agents throughout the natural range of this

plant followed, but no suitable host specific agents were obtained (Harvey & Varley 1996). Recently another indigenous weevil beetle has been found to cause some damage (Creed 1998), but its effect is limited by fish predation (Newman et. al. 2000). Several insects have been released against Hydrilla verticillata, and limited success has been achieved, notwithstanding a herbivorous insect fauna of few suitable candidate agents (Confrancesco et.al. 1996). Low plant quality during autumn is also limiting the success of an insect agent used against H. verticillata (Wheeler & Center 1997).

Therefore despite the successful use of insects against various floating or emergent macrophytes, their use in a submerged situation has been very limited. Most aquatic insects observed feeding on target weeds have been polyphagous, and therefore only indigenous species can be considered for an environmentally safe biological control programme. A number of studies on the interactions of grazing insects or other aquatic animals, and C. glomerata have been carried out. These have been recently reviewed (Joska et.al 2000). Although certain grazers have been shown to have an effect on growth or establishment of C. glomerata, most insects associated with this alga either graze its epiphytes or use it as a shelter only (Whitton 1970). A number of insect larvae were observed to become destructive of laboratory cultures of C. glomerata during a recent survey of potential pathogens in South Africa (Wood 1999). Indigenous insects therefore remain a potential source of biological control agents against this alga.

Fish have long been used for control of aquatic weeds throughout the world. The most commonly used is the grass carp (Ctenophraryngodon idella) (Julien & Griffiths 1998). Results have been varied, but effective control of various aquatic weeds has been achieved with this fish, including in irrigation canals in Egypt (Khattab & El-Gharably, 1989) and Argentina (Armellina et. al. 1999), and lakes in South Africa (Schoonbee, 1991). Recently, because of concerns of the effect of grass carp on indigenous fish species, the use of triploid sterile fish has been investigated. These have been used successfully to control the submerged aquatic plant Hydrilla verticillata in New Zealand (Clayton et. al., 1995). Common carp (Cyprinus carpio) has also been used to control submerged vegetation in drainage canals in Argentina, replacing mechanical removal as the control method (Sidorkewicj et. al. 1998). Triploid sterile grass carp are presently being investigated for use in South Africa as a means of biological control of C. glomerata in irrigation schemes in a Water Research Commission (WRC) funded project being undertaken by Prof. G. Steyn of Rand Afrikaans University, South Africa (Project no. 816).

Two fish species (channel catfish and fathead minnow) indigenous to the Great Lakes, USA, where *C. glomerata* is a nuisance alga, were found to be unsuitable for use as biological control agents against this alga (Patrick *et. al.* 1983).

Because of the success of insects in controlling floating and emergent macrophytes, little work has been done on the use of pathogens for the biological control of these plants. Notwithstanding this, a number of fungi have been proposed for use. Up to the present, no plant pathogen has been successfully used in the classical approach for an aquatic weed, although the rust fungus *Uredo eichorniae* has been investigated for use against water hyacinth (*Eichhornia crassipes*) (Watson 1991, Morris et. al. 1999). The fungus *Cercospora*

piaropi has been introduced into South Africa, but with limited effect on water hyacinth (Morris et. al. 1999).

Several fungal agents have been considered against this and other water weeds for use as mycoherbicides (Charudattan 1991). The fungus Cercospora rodmanii (very closely related to C. piaropi) has been used successfully as a bioherbicide against water hyacinth in Florida, USA. Nutrient levels in water supporting this weed was a critical factor in the effect of this pathogen. At natural nutrient levels the fungus was able to control the weed when applied in an inundative manner (as a mycoherbicide). In contrast, eutrophic conditions allowed rapid compensatory growth of the plant, reducing the effectiveness of the fungus (Charudattan et. al. 1985), and therefore its use was recommended as one measure to be part of an integrated control programme in eutrophic conditions. Alternaria eichhorniae is currently being developed as a bioherbicide for use against water hyacinth (Shabana et. al. 1997, A. den Breeÿen, PPRI Stellenbosch, Personal Communication 2001).

Two fungi have been investigated as potential bioherbicide agents against Eurasian water milfoil (Myriophyllum spicatum). Fusarium sporotrichioides, although capable of causing a serious disease, was dependent on wounds for infection to occur (Andrews & Hecht 1981). Acremonium curvulum was found to be a widespread and common endophyte, which when the plants became stressed, became a serious pathogen (Andrews et. al. 1982). A survey for potential pathogens of M. spicatum in Europe, the plant's native range, identified twelve fungi as potentially pathogenic (Harvey & Varley 1996). None of these fungi have yet to be developed as a bioherbicide. A bacterium causing a wilt disease of M. aquaticum in South Africa was investigated for use as a bioherbicide, but not developed further as it did not cause any sustained damage to the plants (Morris et. al. 1999). Pythium carolianum, originally isolated from the alga Spirogyra, caused a wilt disease of Myriophyllum brasiliense in irrigation ditches. Underwater stems were devoid of rootlets and black cankers were present where the stems were wounded (Bernhardt & Duniway 1984). This fungus was therefore suggested as a potential bioherbicide agent, but has not been developed as such. Pythium species have also been reported as causing disease of Hydrilla verticillata and Lemna spp. (Charudattan 1991). The major impediment in using the above fungi as bioherbicides is the need for wounding or stress of the plants by a third agent before major disease levels could be obtained. They however indicate the potential of fungi to be used indirectly as part of an integrated management programme, whereby a treatment that stresses but does not kill the weed can be applied which would lead to high disease levels and thereby control is achieved.

The fungi Mycoleptodiscus terrestris (Joye 1990 (as Macrophomina phaseolina), Verma & Charudattan 1993), Plectosporium tabacinum (Smither-Koppel et. al. 1999a) and Fusarium culmorum (Smither-Koppel et. al. 1999b) have all shown promise as damaging pathogens against the weed Hydrilla verticillata in laboratory conditions. One of these, M. terrestris, was formulated as a bioherbicide and gave promising results in laboratory experiments (Shearer 1998) and in small scale ponds especially as a dual treatment with the herbicide Fluridone (Nelson et. al. 1998). However in large scale field trials the specifically developed formulation proved to be unsuitable, although the fungus applied in slurry remained highly damaging (Shearer 2000).

Therefore, despite the successful use of pathogens as both classical and bioherbicide agents against terrestrial weeds, their use against aquatic weeds is limited. Fungi used as bioherbicides show a greater potential than if used in the classical approach for aquatic weeds, especially floating weeds that remain under incomplete control by insects such as water hyacinth. The use of fungi, as with insects, appears to have major impediments to their successful utilisation against submerged weeds.

No attempted or successful biological control program of a fresh-water alga using pathogens has been reported on in the world. The author is only aware of one unpublished report detailing an attempt at biological control of Cladophora glomerata using a toxin-producing saprophytic fungus (Acremonium kiliensis) (Patrick et. al. 1983). However an interesting approach to the control of undesirable planktonic cyanobacteria in eutrophic waters has been proposed (Briand & McCauley 1978). In this approach, it is suggested that changes to the balance in biomass between edible and inedible algae (the latter category to which nuisance algae belong, including cyanobacteria) can be manipulated, and favouring edible algae will reduce the levels of nuisance algae. This can be achieved by increasing carnivore (piscivore) populations, or by reducing herbivore or panktivorous predator fish populations. This manipulation increases the biomass of edible algae which reduces the amount of nutrients available to the inedible algae, zooplankton would then remove much of the edible algae. It has been found in a study (Carpenter et. al. 1995) comparing two eutrophic lakes, that one lake with piscivorous fish and large grazers accumulated half the algal biomass as a lake with zooplanktivorous fish and small grazers. Unfortunately blooms of cyanobacteria still occurred in the first lake, although at a reduced level.

Lytic actinomycetes and predatory protozoa have recently been tested under laboratory conditions as potential biocontrol agents against cynanobacteria, with promising results (Sigee et.al. 1999). From practical considerations of using these organisms, the following suggestions were made as regards the optimal use of biological control of cyanobacterial blooms in lakes:

- Early detection of blooms is essential so that control can be achieved early. The quantities
 of inoculum needed to control a well established bloom would be impractical to produce.
- Biocontrol may best be used in small bodies of water (where sufficient inoculum required to treat the water can be easily produced).
- 3. Mixtures of agents, each with a different mechanism of control, may give best results.
- Integrate biological control with other control methods.

These points would apply to any biological control programme in an aquatic setting.

Very little information is available on pathogens of Cladophora glomerata. Whitton (1970, p471) states that "There are apparently no reports of parasites on Cladophora". Yet only ten years previously, Sparrow (1960) listed 24 Chytridiomycete and five Oomycete species as being pathogenic on freshwater Cladophora species. However very little is known about these fungi (see review in Wood 1999).

A previous project (Wood 1999) surveyed for indigenous pathogens of C. glomerata in irrigation canals in the summer rainfall of South Africa. Two Chytridiomycete species (identification uncertain) were commonly observed. However as these are obligate biotrophic

organisms, their direct practical use would be extremely difficult to accomplish. They may still prove to be valuable in indirect use as part of an integrated management programme.

A number of Pythium species were commonly isolated from C. glomerata during this survey, in particular species with non-inflated hyphal sporangia. It has been theorised that the genus Pythium originated as saprophytes in aquatic environments, with most species with noninflated hyphal sporangia (the supposed primitive condition) still leading this lifestyle (Hendrix & Campbell 1983). At least six species (P. adhaerens, P. angustatum, P. apleroticum, P. carolianum, P.dictyosporum, and P.tenue, and a further number of incompletely described species), are known to be pathogenic in a number of fresh water green algae, especially Spirogyra species (Sparrow 1932, van der Plaats-Niterink 1981). Pythium species may also be facultative pathogens of algae. Although only present in less than 1% of total field populations, both Pythium gracile (?) and Saprolegnia asterophora (?) rapidly built up populations under laboratory conditions so that 100% of algal filaments were infected within 3-4 weeks. Notwithstanding this, P. gracile was unable to infect healthy algal filaments in pathogenicity trials, whereas S. asterophora was able to infect (Beck & Erb 1984). The marine red alga Porphyra is also attacked by Pythium species, which can cause serious disease levels during mariculture (Andrews 1976, Kazama 1979). As Pythium species are also well known pathogens of plants, including in hydroponic (soilless) plant growing systems and of some macrophyte aquatic weeds, it was suggested that their potential as biological control agents needs to be investigated, this project was therefore initiated to achieve this goal.

The aims of this project were:

- Investigate the efficacy of selected organisms to control Cladophora glomerata under laboratory and field conditions.
- Investigate the mass production, viable and practical formulations for application of, and the optimal dosage rates of the selected organisms.
- Determine the host range of the selected organisms to ensure crop safety.

The first and third aim were achieved, however as no *Pythium* isolate proved to be highly pathogenic in laboratory conditions, testing under field conditions was not investigated. The second aim was therefore also not pursued.

Additional work was carried out to determine whether the lack of pathogenicity observed was due to environmental conditions, or whether a combination of chemical treatment and inoculation would lead to pathogenicity. The effect of temperature, pH and copper on the growth, zoospore production and zoospore mobility of *Pythium* isolates was investigated. Preliminary studies on the use of *Acacia mearnsii* (black wattle) bark for control of algal growth were carried out. Factors influencing the integration of the use of *Pythium* with other existing or potential control methods were also investigated.

Chapter 2: Materials and Methods

Fresh material of Cladophora glomerata were obtained from the Hartebeespoort (Brits), Mooi River (Potchefstroom), Orange-Riet (Jacobsdal), Roodeplaat (Pretoria) and Vaalhartz (Jan Kemp Dorp) Irrigation Schemes as needed. These were maintained in a growth room at PPRI, Stellenbosch. The algal material was kept in municipal tap water in plastic basins or glass flasks. The water was changed weekly. The cultures were aerated constantly. The growth room had mesh benches (covered with clear Perspex) with four white fluorescent lights above and four white fluorescent lights below. The air temperature was maintained at approximately 25°C.

Stock cultures of isolates previously obtained (Wood 1999) were maintained on Cornmeal Agar (CMA) (extract of 40g of coarsely crushed yellow maize seed, 15g agar per litre distilled water, autoclaved) slants in small bottles kept in a fridge (4°C), and 5mm disks of CMA placed in autoclaved distilled water in small bottles kept in a fridge (4°C). Fresh cultures of isolates were obtained as needed by placing a small sample of the stock cultures onto CMA on 9cm diam. petri dishes, and incubating at 25°C. The cultures in use were maintained by transfer to fresh CMA on a monthly basis or after use.

2.1) Standard method of zoospore production

Zoospores were produced using a standard method (van der Plaats-Niterink 1981) modified as follows. Isolates were grown on water agar (WA) (15g agar per litre distilled water, autoclaved) in 6cm diam. petri dishes with autoclaved pieces of sorghum leaves (WA+L). After four to seven days of growth (25°C) the leaf pieces were removed from the underlying agar and immersed in 10ml soil extract water (SEW) (1g soil per litre distilled water, stirred then left to stand overnight) in 6cm diam. petri dishes. The leaf pieces in water were incubated overnight at 25°C.

2.2) The effect of environmental conditions on Pythium isolates

2.2.1) The effect of temperature on growth

A 5mm diam. plug of each isolate previously obtained (Wood 1999) was transferred to fresh CMA on 9cm diam. petri plates, and the growth measured on two occasions (after 1 {T1} and 2 {T2} days for fast growing isolates, and after 2 {T1} and 4 {T2} days for slow growing isolates). There were two plates per isolate, and the diameter of the colony was measured in two directions perpendicular to each other. The growth rate was determined as:

growth per day (mm) =
$$(\overline{x}T2 - \overline{x}T1)$$
 / no. days

where T1= first measurement at time 1, and T2= second measurement at time 2.

Twelve isolates were selected and their growth rates at 5, 10, 15, 20, and 25°C were measured in the above manner (isolate number 133, 137, 143, 145, 149, 158, 164, 166, 172, 173, 246, 272).

2.2.2) The effect of pH on growth

The growth rates of eight selected isolates were measured at 25°C on CMA adjusted to pH of 5, 6, 7, 8, 9 and 10 with 1M HCl and 1M NaOH using the above method in 2.2.1) (isolate number 133, 137, 143, 149, 164, 166, 172, 173). This trial was repeated once.

2.2.3) The effect of copper on growth

The growth rates of eight selected isolates were measured at 25°C on CMA to which had been added 0, 0.01, 0.05, 0.1, 0.5, 1 and 5 mg Cu⁺/l (as CuSO₄) using the above method (isolate number 133, 137, 143, 149, 164, 166, 172, 173). This trial was repeated once.

2.2.4) The effect of temperature on zoospore production

Eight selected isolates (isolate number 133, 137, 143, 149, 164, 166, 172, 173) were treated as above for zoospore production, except that two 5mm diam. leaf pieces were removed from the WA and immersed in 2ml of a half strength basic nutrient solution (½NS) (stock solution = 40mg MgSO₄.7H₂O, 20mg KCl, 40mg NaHCO₃, 100mg Ca(NO₃)₂.4H₂O, 10mg K₂HPO₄ per litre distilled water, used as 50% stock solution + 50% tap water). There was one bottle per isolate for each of 10, 15, 20, 25 and 30°C incubation temperatures. After 24 hours incubation, one drop of analine blue in lactophenol was added to each bottle to fix and stain the zoospores. Zoospores were counted by means of a haemocytometer. Four drops, and five 0.1mm³ blocks per drop, were counted per bottle, and the average number of zoopores /0.1mm³ calculated.

2.2.5) The effect of pH on zoospore production

The above method in 2.2.4) was used, except that the ½NS was adjusted to pH of 5, 6, 7, 8, 9, 10 using 1M HCl and 1M NaOH and unadjusted control for each isolate, and the bottles were all incubated at 25°C.

2.2.6) The effect of pH and copper on maintenance of zoospore mobility

Zoospores were produced as for the above standard method, using eight selected isolates (isolate number 133, 137, 143, 149, 164, 166, 172, and 173). Initially 1ml of the zoospore solution was added to 1ml of distilled water adjusted to pH6 with 0.2, 0.5, 1, 5 and 10 mg of Cu⁺/l (as CuSO₄), giving final concentrations of 0.1, 0.25, 0.5, 1 and 5 mg Cu⁺/l. This was repeated using concentrations of 0.05, 0.1, 0.2, 0.5, 1 and 2 mg Cu⁺/l giving final concentrations of 0.025, 0.05, 0.1, 0.25, 0.5, 1 and 5 mg Cu⁺/l, and a no copper treatment (0 mg Cu⁺). After 30 minutes, the solutions were observed to determine whether the zoopores were still actively swimming. One drop of analine blue in lactophenol was added to 1ml of zoospore solution of each isolate to fix and stain the spores, and the number of zoospores were counted by means of a haemocytometer. Two drops, and five 0.1mm³ blocks per drop, were counted per bottle, and the average number of zoopores/0.1mm³ calculated.

The same method as above was used except that distilled water adjusted to pH of 5, 6, 7, 8, 9, 10 using 1M HCl and 1M NaOH and an unadjusted control was used, for four selected isolates (isolate number 143, 164, 166 and 172). This test was done twice. Results obtained indicated that distilled water was not a suitable medium for use in these experiments, as it was detrimental to maintenance of mobility.

The above pH treatments were then repeated except that SEW was used instead of distilled water. This gave better results, but was not deemed to be fully suitable. Therefore 1ml of zoospore suspension of seven isolates (the eighth isolate did not produce sufficient spores) was added to 2ml each of distilled water, tap water, SEW, 5g glucose/l distilled water, 10g glucose/l distilled water, ½NS, and full strength NS. After 30 minutes the treatments were observed to determine whether the zoospores had retained their mobility.

From these results, the above pH and copper treatments were repeated twice, using ½NS instead of distilled water.

2.3) Pathogenicity testing towards Cladophora glomerata

2.3.1) Pathogenicity testing of isolates towards Cladophora glomerata

Initially zoospores of 46 isolates of *Pythium* spp. and 9 isolates of *Achyla* spp. obtained from a previous project (Wood 1999) were produced. Ten pieces (approx. 8mm long) of *C. glomerata* that had been pinched with forceps were added to the zoospore solutions, and incubated for 24 hours at 25°C. The algal pieces were examined microscopically and observed as to whether penetration and colonisation of damaged but unbroken cells had occurred or not. This procedure was repeated with 29 isolates of *Pythium* spp., of which seven had not been included in the first lot. Penetration and colonisation of broken and damaged (but still whole) cells was assessed using a simple rating scale: 0 = no penetration, 1 = penetration of broken cells only, and 2 = penetration of broken and damaged cells.

The above method was repeated, except that only 37 isolates of *Pythium* spp. were used. The total number of cells in each algal piece, and the number which had been colonised by each isolate, were counted. The average percent penetrated cells was determined.

For eight selected isolates, the above method was again repeated, except that five of the alga pieces were pinched with forceps, and five pieces were cut with a scalpel causing minimal damage. The average percent penetrated cells was determined. This last trial was repeated once.

2.3.2) Preliminary combination pathogenicity trials

A number of preliminary pathogenicity trials with isolates selected from the above trials were attempted. These included both flask and petri plate trials. In the flask trials, *C. glomerata* cultures (1g wet weight in 250ml glass flasks) were set up in the growth room (see above method). Three separate trials were done.

- In the first, 20 flasks were used to which 3ml of a zoospore solution (combined production of eight selected isolates) was added to half of the flasks. This trial was done twice.
- In the second, ten flasks were treated with water adjusted to pH 6 and another ten to pH 8 for 3 hours, after which a zoospore solution was added to half of the flasks for each pH treatment.
- In the third, 20g/l of fermented barley straw was added to ten flasks, and 200ml/l of fermented barley straw extract was added to ten flasks, and another ten flasks were untreated. A zoospore solution was added to half of the flasks for each treatment. The straw was prepared according to the technique in Gibson et. al. (1990).

In the fourth, 200, 400, 600, 800 and 1000ml of barley straw extract were made up to 1l and dispensed in flasks. There were five replicate flasks per treatment
 In the first trial the flasks were incubated for two weeks. In the second to fourth trials, the flasks were incubated for a week.

In the preliminary trials in petri plates, five alga pieces (approx. 20mm long) were placed in each of thirty two 6cm diam. petri plates with 10ml of ½NS and incubated in the growth room for 7 days. The plates were then treated with the following copper solutions (as CuSO₄, in distilled water adjusted to pH 6): 0, 0.12, 0.25, 0.5, 1, 2.5, 5 and 10mg Cu⁺/l for 2 hours. There were four plates per treatment. After this treatment the algae pieces were resuspended in fresh ½NS. To two petri plates of each treatment, 3ml of a zoospore suspension (combined production of four selected isolates, number 143, 164, 166 and 172) was added, and the plates incubated in the growth room. The alga pieces were observed after four days. This trial was repeated three times. In the first two trials the effect of copper treatment was recorded after one day.

2.4) Pathogenicity testing of crop plants

In an initial trial, sixty isolates of *Pythium* spp., thirteen isolates of *Achyla* sp. and one isolate of *Saprolegnia* sp. were tested in a preliminary trial to determine their potential pathogenicity against crop plants. This was done by the following method:

- 1. All isolates were grown on CMA for 7 days before use.
- 2. Pots filled with soil were autoclaved 3 days before use.
- One pot was used per isolate, in each pot 10 holes of equal depth were made with a dowel rod, and 2 blue lupin seeds and a piece of CMA with the isolate were placed in the holes and covered with soil.
- Pots were kept in a glasshouse and watered regularly.
- Two weeks after sowing the seeds and inoculating them with the isolates, the number of healthy seedlings in each pot was counted.
- 6. Two uninoculated controls were included.
- Three isolates of Pythium from diseased crop plants and of known pathogenicity (with kind thanks to the Soilborne Disease Unit, PPRI Stellenbosch), were included as a comparison.
- The percent surviving plants was calculated for each isolate, the number of plants in the control treatments was taken as 100% for comparison purposes.

This trial was repeated once.

Twelve isolates that showed little pathogenicity in the above initial crop pathogenicity trials, but gave promising results in the first petri plate pathogenicity trials towards *C. glomerata*, were selected and further tested against a range of crop plants that are often grown under irrigation. The crop plants were lupin, sunflower, maize, wheat, tomato, brinjal, pepper, cabbage and lettuce. The same method as above in the initial crop pathogenicity trials was used, except that there were three pots per isolate per crop plant (step 3 in above method). An uninoculated control was included, as were three isolates of *Pythium* from diseased crop plants as a comparison.

2.5) The effect of Acacia mearnsii bark extract on algal growth.

A stock solution of A. mearnsii (blackwattle) bark extract (BBE) was made by placing 403g dry weight of bark in 3l water and decanting the extract after a week.

In preliminary trials, dilutions of the BBE using ½NS were placed in 6cm petri dishes to each of which was added 5 pieces of *C. glomerata*. After three days of exposure the algal strands were examined and it was noted whether they had died, or were healthy and growing. In the first of these preliminary trials the dilutions used were 25, 20, 15, 10, 5, 1, and 0.5%. In the second of these the dilutions used were 5, 1, 0.5, 0.25, 0.1, 0.05, and 0.025%. A control with no extract (0%) was included in each trial. There were two plates per treatment.

Trials were carried out in a similar manner, except that 4 petri plates each with 10 pieces of C. glomerata were used, and the survival rate of all pieces was recorded after 3 days. The concentrations used were 10, 5, 1, 0.5, 0.1% and controls (0%). In addition 2 petri dishes each of Oedogonium sp. and Spirogyra sp. were treated in an identical manner, as were 2 petri dishes each with three pieces of Chalara sp. This trial was repeated once.

The effect of BBE on a larger scale was determined by treating algae (initial wet weight of 2g per flask) in 250ml flasks. The extract was added to ½NS to give final concentrations of 1, 0.5, 0.1, 0.05, and 0.01%, as well as untreated controls. The flasks were incubated in the growth room. Wet weight was recorded 9 and 16 days after the trial was initiated. There were 5 replicate flasks per treatment.

A trial combining BBE and *Pythium* inoculation using flasks was carried out in an identical manner. To ten flasks each was added BBE to give concentrations of 0.1 and 1%, as well as an untreated control. To five flasks of each treatment, 10ml of a zoospore solution (combined production of eight selected isolates) were added. After 8 days incubation in the growth room, the wet weight was again recorded.

Mini canals were constructed in a growth tunnel. These consisted of a length of plastic gutter (2m long) with end pieces placed on a table and filled with water. At one end a hose pipe was attached which allowed the water to flow into a bucket placed below the gutter. An aquarium pump attached to a hose pipe recirculated the water up into the gutter. The total water volume of this system was approx. 20l, and ten systems were constructed. Dry A. mearnsii bark pieces at concentrations of 0, 1, 2.5 and 5g/l were placed in each of two canals. The outer two canals were not used to avoid an edge effect. Three samples of C. glomerata (1g wet weight, in 5x5cm gauze sacks) were placed in each canal, and their wet weight recorded after 14 days.

Chapter 3: Results

3.1) Standard method of zoospore production

Zoospores were readily produced from leaf pieces although different isolates varied greatly in the quantity produced.

3.2) The effect of environmental conditions on Pythium isolates

3.2.1) The effect of temperature on growth

All isolates grew faster at 25°C than at 15°C (Table 1). The results obtained were very similar to those previously obtained, confirming their identity after storage according to the morphological groupings used previously (Wood 1999).

All of the twelve selected isolates (eight fast growing, four slow growing) grew fastest at 25°C. None had reached a growth curve plateau, indicating that their optimal and maximum growth temperature may be higher than 25°C (Figure 1).

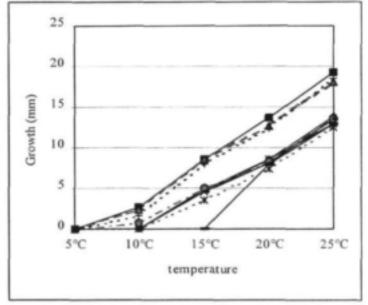


Figure 1. The growth of twelve Pythium isolates at various temperatures on CMA expressed as mm growth per day.

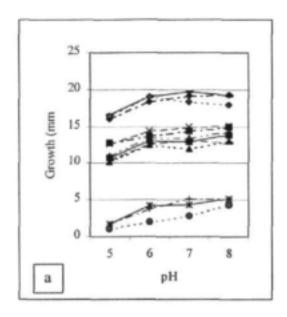
Table 1: Growth of *Pythium* and other isolates at 15 and 25°C per day on Corn Meal Agar. The isolates are placed in morphological groups as determined in Wood 1999.

Group	Isolate	growth	@15°C	growth	@25°C
		19971	2000	1997 ¹	2000
1	133	10	9.2	15.5	15.6
	134	10	9.35	15.5	15.6
	136	10.5	9.4	15.5	16.85
	149	14	13.5	21	20.3
	162	9.5	9.35	16	15.95
	166		8.5		15.7
	173	10	9.85	15	14.8
	283	10	9.1	16	16.2
	284	9.5	8.6	16.5	15.1
	288	10	8	16	15.7
	289	9.5	9.5	16.5	17.15
	290B		8.2		11.6
	311	9.5	9	16.5	14.25
2	265	4.8	4.45	7	9.3
	280	4.7	5.15	7.25	16.4
3	266	4.625	3.25	8.5	7.55
	274	5.375	4.7	9	9.1
	293	5.625	5	9.5	9.4
4	282	10	8.25	17	14.8
	290	10.5	8.55	17	14.4
5	272	10	11.35	15	17.05
6	317	10	7.6	16	15.2
	325	9	7.4	14.5	15.05
7	305	9	10	14	14.85
	312	10	7.85	15.5	14.35
8	313	9		14.5	15.25
	315	9.5	9.35	15	13.5
	316	9		13.5	13.3
9	139	3.125	3.3	4.75	4.8
10	137	8.5	9.3	14.5	13.05
	138	9	7.75	14.5	14.15
	143	8		14	14.05
11	142		12.2		21.7
	165		12.25		20.2
	164	15	12.25	18	20.3

Table 1 continued

Group	Isolate	growth	@15°C	growth	@25°C
		1997^{1}	2000	19971	2000
12	147	2.875	2.55	5	5.6
	156		1.45		3.8
	158	3.25	2.5	5.5	4.4
	159	1.875	2.1	4.5	5.45
	171		2.5		3.85
	245	1.25	1.65	3.875	5.05
	246	1.375	0.9	3.375	2.55
	262	1.375	1.4	3.25	4.2
	264	1.375	2.2	4.625	5.8
	275	0.875	1.45	4.25	4
	287	0.5	1.5	2.875	4.2
	295	1.125	1.45	4.125	3.6
	296	1.125	1.8	3.125	5.3
	297	1.125	1.65	3.25	5.4
	299	1.125	1.95	3.875	5.6
	302	1.125	1.75	3.5	3.45
	303	1.25		3.375	3.85
	304	1.25		3.625	2.9
	307	1.625	1.35	2.625	3.8
	308	1.375	2	4	5.25
	310	1.125	1.7	4	4.85
13	314	2	2.4	3.125	3.8
142	249	11	9.55	16.5	14.8
	250	11.5	9.45	12.5	16.65
	251	11		12	17.05
	252	12	7.7	15.5	15.05
	255	12	10.05	16.5	15.25
	263	11	8.8	16	13.8
	268	11.5	10.5	17	15.8
	269	11.5	10.1	16	15.6
	271	12.5	10.85	17	15.4
	273	11.5	9.1	16	16.1
	276	12	9.6	16	15.6
	277	11.5	9.7	16.5	15.4
	279	12	7.35	15.5	15.45

^{1 =} results presented in Wood 1999 2 = Achyla and Saprolegnia isolates, all other isolates are Pythium species



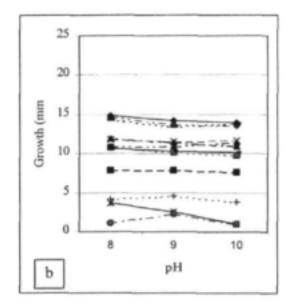


Figure 2: Growth of twelve *Pythium* isolates on CMA adjusted to various pH values at 25°C, expressed as mm growth per day (first trial, fig.2a pH 5–8 and fig.2b pH 8–10)

3.2.2) The effect of pH on growth

Little difference in growth occurred at the various pH tested overall, although growth was slower at pH 5 and/or pH 10 for several individual isolates (Figure 2 & 3).

In the second trial, one isolate had a greatly reduced growth at pH 5 & 6 and two isolates at pH 10, in comparison to the first trial

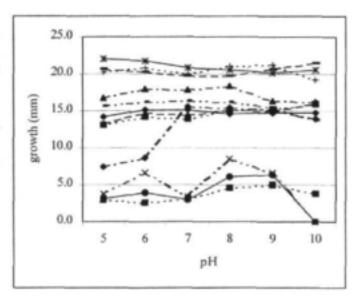
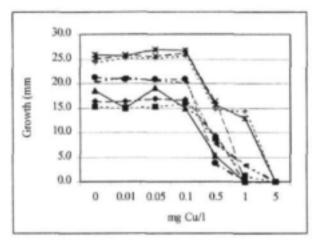


Figure 3: Growth of twelve *Pythium* isolates on CMA adjusted to various pH at 25°C, expressed as mm growth per day (second trial)

3.2.3) The effect of copper on growth



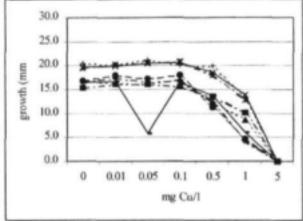


Figure 4: Growth of eight *Pythium* isolates on CMA amended with Cu (as CuSO₄) at 25°C, expressed as mm growth per day (first trial)

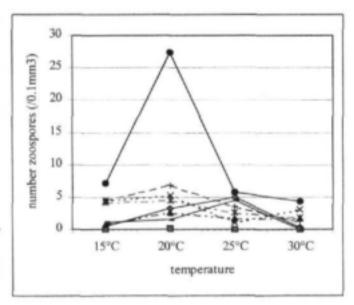
Figure 5: Growth of eight *Pythium* isolates on CMA amended with Cu (as CuSO₄) at 25°C, expressed as mm growth per day (second trial)

The growth of the *Pythium* isolates were unaffected at copper concentrations of ≤ 0.1 mg /l. Growth was reduced at 0.5mg/l. The lethal dose was 1 or 5mg/l depending on the isolate in the first replicate of this trial (Figure 4), but only 5mg/l in the repeat trial (Figure 5).

The effect of temperature on zoospore production

The optimum temperature for zoospore production was either 20 (6 isolates) or 25°C (2 isolates), depending on isolate (Figure 6).

Figure 6: Average number of zoopores of eight *Pythium* isolates produced per 0.1 mm³ at different temperatures.



3.2.3) The effect of pH on zoospore production

The results are variable, with no consistent trends within the two trials. In the first trial, alkaline conditions (pH 8 – 10) favoured zoospore production. The greatest production occurring at pH 10 (4 isolates). In only one isolate was acidic pH of 5 & 6 slightly more favourable than alkaline pH (Figure 7).

In the second trial, some isolates had greater zoospore production at acidic pH (3 isolates), whilst others had greater production at alkaline pH (5 isolates). Only two isolates had maximum production at pH 10 (Figure 8).

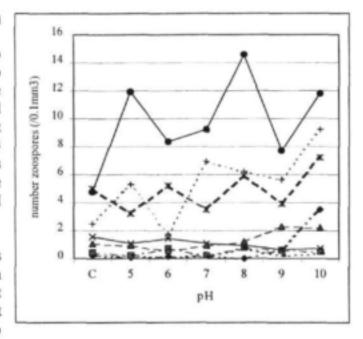


Figure 7: Average number of zoospores of eight Pythium isolates produced per 0.1mm³ in soil extract water adjusted to various pHs, at 25°C after one day.

A far higher level of zoospore production was recorded in the first trial (using SEW) than in the second trial (using ½NS).

There was no consistency as to relative quantity of zoospore production within one isolate between the two trials.

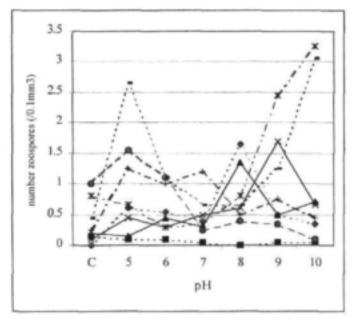


Figure 8: Average number of zoospores of eight Pythium isolates produced per 0.1mm³ in nutrient solution adjusted to various pH, at 25°C after one day.

3.2.6) The effect of pH and copper on maintenance of zoospore mobility.
In the preliminary trials of various copper concentrations using distilled water, no zoospores remained active at any concentration (see Table 2).

Table 2: The effect of copper concentrations on *Pythium* zoospore mobility, using distilled water adjusted to pH 6.

isolate	No. spores	Susp.2	04	5	2.5	1	0.5	0.25	0.1	0.05	0.025
133	1.0	y ³	ND_2	-	-	-	-	-	-	ND	ND
137	1.0	y	ND	-		-	-	-	-	ND	ND
143	1.4	У	ND	-	-	-	-	-	-	ND	ND
149	0.9	У	ND	-	-	-	-	-	-	ND	ND
164	2.1	y	ND	-	-	-	-	-	-	ND	ND
166	1.8	У	ND	-	100	-	-	-	-	ND	ND
172	3.3	y	ND	-		-	-	-	-	ND	ND
173	0.3	-	ND	-	-	-	-	-	-	ND	ND
133	1.1	У	У	ND	ND	-	-	-	-	-	-
137	2.6	У	У	ND	ND	-	-	-	-	-	-
143	5.1	y	y	ND	ND	-	-		-	-	-
149	6.1	У	У	ND	ND	-	-	-	-	-	-
164	4.1	y	-	ND	ND	-	-	-	-	-	-
166	4.6	У	y	ND	ND	-	-	-	-	-	-
172	6.9	y	y	ND	ND	-	-	-	-	-	-
173	0.6	У	-	ND	ND	-	-	-	-	-	-

¹⁼ average number of zoospores/0.1mm³

In the preliminary trials with distilled water adjusted to various pH, the results were variable but clearly demonstrated firstly that distilled water itself is not suitable for maintaining zoospore mobility, and that alkaline conditions are conducive to maintaining zoospore mobility (Table 3). The trial was repeated using soil extract (to provide nutrients) instead of distilled water, with similar results (Table 4).

Because of the low levels of zoospore mobility in the controls of the above trials, the trial comparing various nutrient solutions was therefore carried out. The results demonstrate that nutrients are necessary for the maintenance of zoospore mobility, with ½NS being the best, followed closely by soil extract (Table 5).

²⁼ untreated zoospore suspension

³⁼ y: zoospores remained mobile, -: no zoospores still swimming

⁴⁼ mg Cu⁺/l, in the form of CuSO₄

⁵⁼ Not Done

Table 3: The effect of pH on Pythium zoospore mobility in distilled water adjusted to various pHs.

isolate	No. spores	Susp.	pH 5	pH 6	pH 7	pH 8	pH 9	pH 10	dH ₂ O
143	3.6	у	У	у	У	у	У	ND	ND
164	2.2	У	-	-	y	у	У	ND	ND
166	5.7	y	y	y	у	y	У	ND	ND
172	6	у	-	y	-	у	У	ND	ND
143	0.8	У		-	У	у	У	у	у
164	1.9	y	-		-	у	y	y	-
166	1.7	у	-	-	-	y	У	y	
172	2.2	у	-	-	-	у	У	У	-

1= unadjusted distilled water control

See Table 2 for explanations

Table 4: The effect of pH on Pythium zoospore mobility in soil extract water adjusted to various pHs.

isolate	No. spores	Susp.	pH 5	pH 6	pH 7	pH 8	pH 9	pH 10	SEW
133	1.1	У	-	у	У	у	У	у	у
137	1.3	y	y	y	-	у	y	y	y
143	6.3	У	-	y	y	y	У	У	У
149	1.3	-	-	-	y	y	у	У	-
164	2.3	y	-	у	У	У	У	y	-
166	5.4	У	y	У	У	-	У	У	y
172	5.5	У	-	-	у	y	у	y	У
173	5.0	У		-	y	У	У	y	-

1= unadjusted soil extract water control

See Table 2 for explanations

Table 5: The effect of various nutrient treatments on Pythium zoospore mobility.

isolate	No. spores	Susp.	dH ₂ O ¹	tap	SEW	5g G	10g G	½NS	NS
133	1.0	У	-	-	у	-	-	У	-
137	1.2	y	y	-	y	-	y	У	У
143	1.3	У	-	-	У	y	у	У	у
149	3.1	y	y	-	y	y	У	y	-
166	3.5	y	-	-	y	y	-	y	y
172	4.6	У	y	-	У	У	y	y	У
173	7	y	-	-	y	-	-	y	y

1 = The treatments are distilled water, tap water, soil extract water, 5g glucose/l dH₂O,

10g glucose/l dH2O, 1/2NS medium, and full strength NS respectively.

See Table 2 for explanations

Using ½NS, the effect of pH was better demonstrated than in the initial trials using distilled water (Table 6). At the acidic treatments (pH 5 & 6), zoospore mobility was either stopped or reduced. In all cases where there were still mobile zoospores observed at pH 5 and 6, the number of these were noticeably less than the number in the controls and higher pH treatments. In some cases, pH 10 also either stopped or reduced zoospore mobility.

Table 6: The effect of pH on Pythium zoospore mobility in ½NS adjusted to various pH. The trial was repeated three times.

isolate	No. spores	Susp.	pH 5	pH 6	pH 7	pH 8	pH 9	pH 10	⅓NS
133	4.1	у	-	У	У	У	У	у	У
137	3.5	У	-	У	y	y	У	У	У
143	3.2	y	-	y	y	y	y	y	y
149	2.1	У	-	y	y	y	У	-	y
164	1.1	y	-	y	y	y	-	У	-
166	5.1	y	-	У	У	У	y	У	y
172	8.2	y	-	У	y	y	У	y	y
173	3.5	У	-	y	У	y	y	У	У
133	0.1	у	У	у	у	у	У	-	У
137	0.5	y	У	У	y	у	У	take .	У
143	0.6	y	-	-	У	y	У	-	-
149	6	y	у	У	У	y	-	-	y
164	8.6	У	У	y	У	У	y	У	У
166	0.4	У	y	y	У	y	У	_	У
172	5.7	У	y	У	У	y	У	-	У
173	0.4	У	y	y	У	y	У	У	-
133	1	У	У	у	У	у	у	У	У
137	0.3	y	y	У	У	y	У	У	У
143	1.1	y	У	y	-	y	_	y	У
149	7.4	У	_	У	у	У	y	у	У
164	3.5	У	-	y	У	y	y	У	y
166	1	У	-	y	У	y	У	у	У
172	2.3	y	-	у	у	y	У	у	У
173	3.3	У	y	y	У	y	y	У	У

See Table 2 for explanations

The zoospores were highly sensitive to copper treatment (Table 7), only at the lowest concentration used (0.025mg/l) was zoospore mobility not affected by copper. In two cases (isolate number 137 and 143) the fungi may be slightly less sensitive to copper than the other isolates. In the two cases (isolate number 164 and 172) where there was still mobility at higher concentrations (0.1mg/l), these were when there had been a very high zoospore production.

Table 7: The effect of copper concentrations on *Pythium* zoospore mobility, using half strength nutrient solution adjusted to pH 6. The trial was repeated twice.

isolate	No. spores	Susp.	0	1	0.5	0.25	0.1	0.05	0.025
133	2.2	у	У	-	-	-	-	-	У
137	2.6	y	y	-	100	-	-	y	y
143	4.5	У	y	-	-	-	-	-	У
149	1.1	У	У	-	-	-	-	-	_
164	1	У	У	-	-	-	-	-	y
166	2.4	У	y	-	-	-	-	-	У
172	3.8	y	y	-	-	-	-	-	_
173	0.7	У	y	-	-	-	-	-	У
133	0.8	у	У	-	-	-	-	-	У
137	0.7	y	y	-	-	-	-	y	y
143	2.4	У	У	-	-	-	-	У	У
149	14.1	y	y	-	-	-	-	-	У
164	15.5	y	y	-	-	-	y	y	У
166	0.3	-	У	-	-	-	-	-	_
172	18.3	y	y	-			У	y	У
173	3.5	y	У	-	-	-	_	-	У

See Table 2 for explanations

3.3) Pathogenicity testing towards Cladophora glomerata

3.3.1) Pathogenicity testing of isolates towards Cladophora glomerata

The initial pathogenicity testing was promising, in that isolates of certain *Pythium* morphological groups readily formed appressoria on damaged but unbroken cells of *C. glomerata*, penetrated through the walls and colonised the cell (Table 8). This was consistent through all three repeats of this trial.

After the disappointing results obtained in the preliminary combination pathogenicity trials (see below), the trial comparing pathogenicity of selected isolates to damaged (pinched) and undamaged (cut) algal pieces was carried out. Results of this trial (Table 9) show conclusively that *Pythium* is only able to attack damaged cells. The percent of colonised cells is a reflection of the number of cells damaged by the forceps rather than a measure of pathogenicity. The cells colonised in the undamaged treatment were usually on an end, and reflect the few cells where each algal piece was held by forceps during transfer to the petri plate, and hence were slightly damaged though not broken.

Table 8: Pathogenicity of *Pythium* isolates to damaged (pinched with forceps) pieces of *Cladophora glomerata* (10 pieces alga per isolate per trial, each algal piece approx. 10mm long and pinched at least once in the middle). In the first two trials, a simple rating scale was used to assess damage, in the third the percentage of cells penetrated was determined. The isolates are separated into morphological groups. Not all isolates were included in each trial.

Group	isolate	trial 1*	trial 2*	trial 3 ⁸	Group	isolate	trial 1 ^	trial 2 "	trial 3 ⁸
1	133			11.6	12	145		2	37.2
	134	1		15		147		0	
	136	0		2.9	l	156		1	
	149	1	2	25	l	158		2	21
	162	1	2	12.8	l	159	1		
	166	0		24.3	1	171	0		
	172	2	2	21.1	l	245	2		
	173	0	2	25.7	l	246	0		42.6
	283			13.5	l	264	0	0	
	284		2	12.5	l	275	1		
	288	2	2	17.9	1	287	1	2	
	289			24.7	l	296	0	0	
	311	1	2	14.8	l	297	0	0	
3	266	0		0	1	299	1	0	
	274	0		0	1	302	0	0	
	293	0		0	l	303	0	0	17.6
4	282	0		18.7	1	307	1		
	290	0	0	12.6	l	308	0		
5	248	0	0	16.2	1	310	1		
	272	1	1	14.7	13	314	1		_
6	317	1	2	14.7	14	249	0		0
	325	1	2	12.3		250	0		0
7	305	2	2	12.7	1	251	0		0
	312	1	2	18.5	1	252			0
8	313	0		5	1	255			0
	315	0		0		263	0		
	316	0		1.3	1	268	0		0
9	139		2		1	269	0		0
10	137	0		19.3	1	271	-		0
	138			13.8		273	0		0
	143	2	2	17.2		276	0		0
11	142	-	0	21.8	-	277	0		1.8
	165	1	U	3.6		279	0		0
	164	2	2	14.8		2/0	-		- 0

A rating scale: 0 = no penetration or growth on spilt contents only, 1 = penetration of broken cells only by means of appressoria, 2 = penetration of damaged (but still whole) cells by means of appressoria.

^B average percent of cells in strands of *C. glomerata* penetrated and colonised by each *Pythium* isolate.

Table 9: Average percent penetrated cells of *Cladophora* glomerata pieces by *Pythium* species. There were five algal pieces (approx. 8mm long) damaged by pinching with forceps, and five pieces undamaged. Two replicate trials were carried out.

Isolate		ted cells in	% penetrated cells in Undamaged pieces			
	trial 1	trial 2	trial 1	trial 2		
control	0		1.3			
133	28.3	45.9	2.4	12.0		
137	22.7	43.6	3.0	9.6		
143	55.5	28.3	7.5	7.5		
149	38.3	34.3	7.5	10.0		
164	46.7	26.5	6.3	2.3		
166	40.8	42.2	5.4	9.7		
172	43.0	51.1	11.5	3.1		
173	37.1	32.7	1.6	4.3		

3.3.2) Preliminary combination pathogenicity trials

In the first flask trials where half the flasks were inoculated, no differences were observed between the inoculated and uninoculated flasks. No final weighing in these cases was done as the algal contents of all the flasks had collapsed at the end of a two-week period. It was observed that a tremendous amount of epiphytes and contaminant algae were present in the flasks, as was a build up of chytrid pathogens.

In the second flask trial where half the flasks were treated at pH 6 and the other half at pH 8, and half of each of these were inoculated, the growth was very variable between flasks within each treatment. No differences between any of the treatments were therefore observed (Table 10).

In the third flask trial where fermented barley straw and fermented barley straw extract was added to one third of the flasks, and half of each was inoculated, growth was less for the inoculated control and extract treatments than the uninoculated corresponding treatments. However the variation within each treatment was high (Table 11).

In the fourth flask trial where fermented barley straw extract was added in various concentrations to flasks, no differences occurred between each treatment (Table 12).

Table 10: Average percent growth (±SE) of algal samples one week after treatment at pH of 6 or 8, and inoculated or not with *Pythium* isolates

pH	% growth in	% growth in	
	uninoculated	inoculated	
6	-17.2 (±5.6)	-7.4 (±9.2)	
8	-18.3 (±20.5)	-21.6 (±13.1)	

Table 11: Average percent growth (±SE) of algal samples two weeks after treatment with barley straw or extract, and inoculated or not with Pythium isolates.

treatment	% growth in uninoculated	% growth in inoculated	
control	9.4 (±13)	0.4 (±4.6)	
barley straw (20g/l)	-5.6 (±2.8)	8.2 (±3)	
extract (200ml/l)	22.4 (±3.9)	9.8 (±4.7)	

Table 12: Average percent growth (±SE) of algal samples two weeks after treatment with barley straw extract.

treatment	% growth	
control	-13.6 (±1.9)	
extract (200ml/l)	$-3.6 (\pm 2.3)$	
extract (400ml/l)	$-3.4 (\pm 4.9)$	
extract (600ml/l)	-13.2 (±7.5)	
extract (800ml/l)	-10.8 (±3.7)	
extract (1000ml/l)	-11.4 (±4.5)	

Table 13: The effect of copper concentration on Cladophora glomerata

copper concentration (mg/l)	trial 1	trial 2
0 (control)	no chlorosis	no chlorosis
0.125	no chlorosis	no chlorosis
0.25	no chlorosis	no chlorosis
0.5	no chlorosis	no chlorosis
1	no chlorosis	few cells chlorotic
2.5	few cells chlorotic	most cells chlorotic
5	all cells chlorotic	all cells chlorotic
10	all cells chlorotic	all cells chlorotic

In the petri plate trials where the combination of copper treatment and inoculation with Pythium zoospores was attempted, C. glomerata was killed at a concentration of 5 mg Cu/l, and detrimentally affected at 2.5 mg Cu/l (Table 13). However, no differences were noted at any concentration between the inoculated and uninoculated algae in all three trials.

3.4) Pathogenicity testing of crop plants

The majority of isolates showed no pathogenicity to blue lupin, a crop plant that is highly susceptible to *Pythium*. Only 5 isolates showed a low pathogenicity to blue lupin in both trials, although others had a low pathogenicity in one but not the other trial. There was no consistency within the morphological groups as to pathogenicity (Table 14).

None of the twelve isolates selected from the initial *Cladophora* pathogenicity trial (above) were consistently pathogenic in these crop plant trials. However a few did show a low level of pathogenicity in at least one of the two replicates.

The various crop plants differed greatly in their overall susceptibility to the isolates (Table 15). Lupin, sunflower and lettuce showed a general low susceptibility overall. Maize wheat, tomato, brinjal, green pepper and cabbage were all highly susceptible to the three comparison isolates of known pathogenicity to crop plants. Three of the twelve tested isolates were pathogenic or highly pathogenic to 5 of the crop plants (149, 164 and 172).

Table 14: Initial crop pathogenicity trial, results are expressed as percent survival of 20 blue lupin seeds compared to the control, the trial was repeated twice. Treatments with less than 70% survival are shaded.

isolate	Pathoge	nicity	1	isolate	Path	ogenicity
	% of co	ontrol			% (of control
133	91	60		145		84
134	74	103		147	80	60
136	91	103		156	97	66
149	68	84		158	85	54
162	85	96		159	85	
164	91	90		171	97	90
166	85	66		178	9	66
173	85	103		245	51	66
172	630	66		246	80	72
283	74	103		262	85	103
284	108	78		264	74	90
288	91	72		275	51	78
289	74	84		287	91	
290B	85			295	68	96
311	80	90		296	97	
				297	102	78
265	97	103		299	80	96
280	68	90		302	85	90
				303	85	72
266	97	90		304	97	78
274	85	84		307	80	84
293	102	103		308	68	78
				310	62	
282	91	96				
290	57	90				
				249	85	96
248		96	ł	250	97	96
272	91	90		251	114	103
				252	85	60
317	91	96		255	68	78
325	91	96		263	85	90
				268	97	72
305	91	90		269	74	54
312	68	78		271	80	66
				273	85	90
313	91	96	1	276	85	96
315	97	60		277	85	90
316	97	66		279	51	90
139	91		l	314	97	72
137	85	90	l			
138	80	96	l	control (1)	100	100
143	80	60	l	control (2)	100	100
	-	Page 1 dis	}	isolate W92h	34	48
142	97	72		isolate W60f	40	42
165	14	84	l	isolate W60i	74	66
						23

Table 15: Percent survival of seedlings of various crop plants to inoculation with selected *Pythium* isolates, in comparison to the controls (treatments with less than 60% survival are shaded grey).

isolate	lupin	sunfl.	maize	wheat	tomato	brinjal	pepper	cabb.	lettuce
control	100	100	100	100	100	100	100	100	100
W60I	91	94	23	12	12	4	2	2	95
W60F	80	94	31	15	3	13	2	0	103
W92H	58	96	7	5	3	0	0	35	70
133	91	87	92	88	120	76	108	60	108
137	96	101	68	81	63	46	44	75	97
143	96	100	94	90	148	41	88	92	105
145	96	101	104	98	168	88	64	107	111
149	91	96	57	70	12	0	0	47	70
158	96	101	120	61	120	79	35	105	59
164	86	101	49	92	0	0	20	27	67
166	98	100	68	96	189	111	132	80	83
172	63	97	76	66	3	0	0	22	43
173	96	101	88	96	120	74	55	75	108
246	93	101	110	105	153	125	94	92	
272	93	101	96	88	108	65	44	80	113

3.5) The effect of Acacia mearnsii bark extract on algal growth All C. glomerata pieces in concentrations of 10, 5, 1, and 0.5% (except for 3 pieces in one plate at 0.5%) died. In contrast, rapid growth occurred at 0.1% and in the controls (Table 16).

Identical results were obtained with *Oedogonium* sp. *Spirogyra* sp. was more tolerant of treatment. In the first trial mortality only occurred at 10, 5, and 1% and in the second trial only at 10%. However many individual cells were killed at 1%, and the chloroplasts were completely disrupted though still green in all cells at 5%. Growth was inhibited at 0.5% in both trials, although no mortality was recorded. Rapid growth occurred at 0.1% and in the controls. At a 1% concentration the tips of the *Chalara* sp. branches only were still green, the rest of the pieces were dead.

Table 16: Survival rates of four species of algae in different concentrations of *Acacia mearnsii* bark extract, after three days of exposure. The number of surviving algal strands out of the 10 original ones (or 3 in the case of *Chalara*) is given. The trial was repeated, but excluding *Chalara*.

conc. (%)	Cladophora	Oedogonium	Spirogyra	Chalara
10	0, 0, 0, 0	0, 0	0, 0	0, 0
5	0, 0, 0, 0	0, 0	0, 0	0, 0
1	0, 0, 0, 0	0,0	0,0	3, 3
0.5	0, 0, 0, 0	0, 0	10,0	3, 3
0.1	10, 10, 10, 10	10, 10	10, 10	3, 3
0	10, 10, 10, 10	10, 10	10, 10	3, 3
10	0, 0, 0, 0	0, 0	0, 0	
5	0, 0, 0, 0	0,0	0,0	
1	0, 0, 0, 0	0,0	5, 5	
0.5	0, 0, 0, 0	0,0	10, 10	
0.1	10, 10, 10, 10	10, 10	10, 10	
0	10, 10, 10, 10	10, 10	10, 10	

In the trial on the effect of BBE in flasks, only at 1% was mortality recorded, however growth was inhibited in comparison to the control at all other concentrations (Figure 9). The controls started to degenerate due to a heavy epiphyte and contaminant load at the end of the trial, giving a lower than expected wet weight. In contrast there was little contamination of the algae in the 0.05 and 0.01% concentrations, although growth was inhibited. No difference in growth occurred between inoculated and uninoculated flasks with different percentages of BBE (Figure 10).

In the mini canal trial, treatment with black wattle bark caused a reduction in wet weight (Table 17).

Table 17: Growth (±SE) of Cladophora glomerata in mini canal systems amended with different concentrations of Acacia mearnsii bark (initial wet weight = 1.0g).

Concentration (g bark/l)	Final wet weight (g)
0 (control)	1.2 (±0.031)
1	1.1 (±0.176)
2.5	$0.8 (\pm 0.052)$
5	0.8 (±0.040)

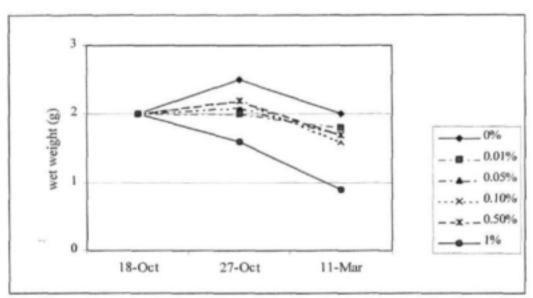


Figure 9: Change in wet weight of Cladophora glomerata samples after 9 and 24 days in $\frac{1}{2}$ NS amended with various concentrations of an Acacia mearnsii bark extract ($1\% \approx 1.4$ g dry wt. bark/l, initial wet weight = 2.0 g).

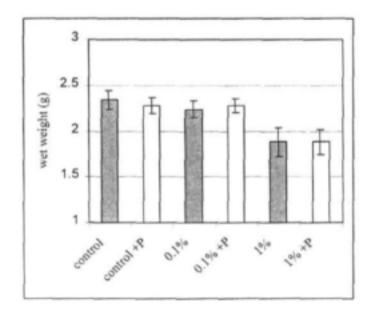


Figure 10: Average growth (±SE) of Cladophora glomerata samples after 9 days in ½NS amended with two concentrations of an Acacia mearnsii bark extract, with (+P) or without inoculation with a Pythium zoospore solution (initial wet weight = 2.0 g).

Chapter 4: Discussion

At the Orange-Riet Irrigation scheme from January 1995 to July 1997, temperatures ranged from 8 to 25°C with temperatures at the low end during winter and at the upper end during summer, and pH from 6 to 9 but mostly 7 to 8 (Joska et. al. 2000). These figures are more or less applicable for all the irrigation schemes from which the *Pythium* isolates had been previously obtained (Wood 1999). The growth of the *Pythium* isolates fits within these parameters, with fastest growth occurring at 25°C (the optimal temperature may be higher but further testing was not done), and at pH of 7 to 9. Similar results were obtained for zoospore production, and with the effect of pH on zoospore motility.

A standard dosage of 0.5 mg Cu⁺/l (as CuSO₄) after adjusting the water pH to 6, is currently employed to control C. glomerata in the irrigation canals of various irrigation schemes (du Plessis & Donaldson 1996). This dosage was found to slow the growth of selected Pythium isolates on CMA, but was not lethal. No growth occurred at 1 or 5 mg/l (depending on isolate). Similarly C. glomerata itself was found to be killed at 2.5 or 5 mg/l in petri dish trials. This result is confirmed by previous laboratory experiments (results presented and literature cited in Joska et. al. 2000), where 2 mg/l or higher is the minimum copper concentration required to control growth. The disparity between the experimental results and practical experience in the field is intriguing and deserves further investigation.

Zoospores were extremely sensitive to copper, as shown by the zoospore mobility experiments. Only at the lowest concentration tested, 0.025 mg/l, was zoospore mobility not stopped by copper treatment. pH also played a significant role, as did nutrient levels, in maintaining zoospore mobility.

Initial results of pathogenicity testing against C. glomerata with pieces of wounded (pinched with forceps) algae gave very promising results. Wounded but unbroken cells were readily penetrated en masse by a number of isolates. Unfortunately when these tests were repeated with unwounded (cut) algal pieces, no pathogenic isolates capable of causing death were observed. The wounded algal cells were readily colonised but the unwounded were not. Therefore none of the Pythium isolates tested would be suitable for development as a bioherbicide for the control of C. glomerata. This does not preclude these fungi, together with the chytrid fungi observed in the same systems (Wood 1999), playing an important indirect role in an integrated control programme. The widespread indigenous endophytic fungus Acremonium curvulum became a destructive pathogen only when the host Myriophyllum spicatum was stressed (Andrews et. al. 1982). The chytrids Zygorhizidium affluens (Canter & Lund 1951 (as Rhizophydium planktonicum), Kudoh & Takahashi 1990) and Z. planktonicum (van Donk & Ringelberg 1983) pathogenic to the diatom Asterionella formosa, although always present in the host populations, reduced the abundance of its host only when the latter was under some environmental stress such as unfavourable temperatures. These two examples demonstrate the potential that fungi have to be an indirect part of an integrated control programme where some environmental factor is manipulated rather than the direct application of a mycoherbicide. Aquatic algae eating insects would also contribute to achieving control in such a programme.

Selected isolates also showed no pathogenicity in preliminary trials where unwounded algae were inoculated in combination with pH, barley straw or its extract, copper, and black wattle bark extract, treatments.

Pythium species were readily isolated from algae collected on most occasions and from all irrigation schemes visited during the survey for potential pathogens. In addition on a number of occasions fungal hyphae were observed in still-alive algal cells which had greatly reduced chloroplast numbers. This chlorosis extended for several cells and generally at one side dead cells still with hyphae were observed, suggesting that the fungus had killed or was in the process of killing cells. It was assumed that these hyphae belonged to at least one of the Pythium species obtained (Wood 1999). The above results suggest that the fungus causing the disease is not a Pythium species, and therefore further efforts to obtain, isolate and identify this specific pathogen is suggested. The potential for a pathogen to be found and developed as a mycoherbicide, even though the Pythium species studied in this project were found to be unsuitable, is still present.

In general, the *Pythium* isolates proved to be non-pathogenic or only slightly pathogenic to lupin, only one isolate caused less than 70% crop survival in both of the initial trials. However, in the pathogenicity trial of selected isolates to various crop plants, three of the twelve isolates proved to be highly pathogenic, as were the three comparison isolates. It is interesting to note that of the three comparison isolates (isolated from crop plants and known to be pathogenic), two generally caused far higher disease levels than the aquatic isolates tested in this work. The third comparison isolate, known as a weak pathogen, caused disease approximately equivalent to that of the tested isolates which showed some pathogenicity. Although these trials are designed to favour disease development, and are not necessarily a true reflection of the disease levels encountered under field conditions, they still show that some of the aquatic *Pythium* species present in the irrigation schemes are capable of causing disease to certain crop plants over which they may be irrigated. This would preclude the mass production and release of these specific isolates into irrigation schemes as a mycoherbicide.

Barley straw has been reported as controlling C. glomerata in canals (Welch et. al. 1990), as well as other algae (e.g. Everall & Lees 1996, Harriman et. al. 1997, Welch et. al. 1990). However in the preliminary trial reported on here, and some preliminary work by Joska et. al. (2000), local (South African) barley straw does not appear to have any effect on growth of C. glomerata. It has been postulated that the active ingredient is oxidised polyphenolics from solubilised lignin or tannins released by decomposition (Ridge & Pilinger 1996). Tannins derived from various plants have also been reported as controlling algae in laboratory studies (Hussein Ayoub & Yankov 1985). The use of Acacia mearnsii (Black wattle) bark, a readily available resource in South Africa, was investigated to determine if it could be a suitable alternative control method. Results showed that it was algicidal at concentrations of approximately 1g bark/l, and inhibited

growth at lower concentrations. The use of black wattle bark deserves to be further investigated, including detailed toxicological studies to determine whether negative environmental effects occur.

The aims of the project were:

- Investigate the efficacy of selected organisms to control Cladophora glomerata under laboratory and field conditions.
- Investigate the mass production, viable and practical formulations for application of, and the optimal dosage rates of the selected organisms.
- 3. Determine the host range of the selected organisms to ensure crop safety.

The first and third aim were achieved, however as no *Pythium* isolate proved to be highly pathogenic in laboratory conditions, testing under field conditions was not investigated. The second aim was therefore also not pursued.

Environmental conditions (temperature, pH and copper) affecting growth and zoospore production by *Pythium* isolates, and factors influencing the integration of the use of *Pythium* with other existing or potential control methods were also investigated, after discussion with the Steering Committee. Preliminary studies on the use of *Acacia mearnsii* (black wattle) bark for the control of algal growth were also carried out

Chapter 5: Conclusions

Conclusions reached from the work done were:

- The Pythium isolates tested are adapted to the conditions from which they were isolated.
- Copper treatment at 0.5 mg Cu⁺/l is unlikely to kill all the Pythium mycelium present in a system, but it will reduce the potential inoculum and in particular all zoospores will be eliminated.
- 3. The Pythium species tested proved to be incapable of penetrating healthy algal cells, and therefore can't be considered as pathogens with the potential to be developed as a bioherbicide. These fungi are not the causal organism of diseased C. glomerata observed previously, and the use of Pythium species as biocontrol agents against C. glomerata will not be pursued further.
- A few isolates of Pythium proved to have a high potential pathogenicity against certain of the crop plants tested.
- Acacia mearnsii bark proved to be algicidal (at high concentrations) or to inhibit growth (at low concentrations) of C. glomerata.
- Fungal pathogens other than Pythium are present in the irrigation schemes and may yet prove to be valuable biocontrol agents against C. glomerata.

Chapter 6: Recommendations

The following general observations can be made concerning problematic algae (including Cladophora glomerata) in irrigation schemes in South Africa:

- Eutrophication is one cause of the increasing problem of algae in irrigation schemes, and efforts to reduce eutrophication need to be implemented. Such efforts will limit, but not eradicate, the problem of algal blooms in the irrigation schemes. Also increased algal blooms play a role in reducing the effects of eutrophication on water quality, and this role should be assessed.
- At present control of algae in the irrigation canals is carried out in reaction to large blooms. A paradigm shift in management is required to an Integrated Pest Management (IPM) programme, involving monitoring of environmental conditions and early stages in the build up of algal populations, and then proactive control measures being implemented.
- 3. The appropriate use of copper treatment, mechanical removal of algal mats, canal cleaning, water level fluctuations, emptying of water in selected canals, herbivorous fish, and biological control, needs to be determined for each irrigation scheme and individual canals within each scheme. Each scheme needs a detailed plan with a range of control measures applied at different times to different canals, applying measures appropriate to each situation.

Within a framework of an IPM programme, the following recommendations emanating from this project are:

- The role of organisms (insects and fungi) in reducing algal biomass after a stress treatment such as fluctuating the water level or a low dose of an algaecide needs to be determined.
- The short and long term effects of copper treatment on organisms capable of attacking Cladophora glomerata need to be assessed, and their recruitment rates after copper treatment determined.
- The potential of Acacia mearnsii bark to control algal blooms needs to be assessed, as well as possible toxicological effects.
- Further efforts to isolate the observed pathogen of Cladophora glomerata present in at least some irrigation schemes need to be carried out, and its potential as a biological control agent tested.

Integration of copper treatment with any biological processes will be problematical due to the general toxicity of the chemical to microorganisms. Environmentally safe alternatives need to be sought, although it is recognised that there is a paucity of such chemicals – a worldwide problem where managers need to balance needs of water users and environmental concerns.

The indirect use of *Pythium* species and other pathogens (such as the chytrids known to be common) present in the irrigation schemes, after treatments such as water level fluctuation or dosing with low concentrations of *A. mearnsii* bark, may prove to be a valuable process within an IPM programme. Further searching for an unidentified pathogenic fungus known to be present but not isolated may also still prove to be a fruitful direction for future research, with the prospect of a mycoherbicide being developed as a possible future achievement.

Worldwide Cladophora glomerata remains a management problem in waterways, with chemical control options diminishing due to environmental concerns. The process of developing viable and suitable control options in South Africa is important locally and internationally. This project only formed a part of this process, there is great need for the whole process to continue.

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Two problem species of filamentous algae were identified. *Oedogonium capillare* occurred in the lower pH water in the canals of the Breede River System, and *Cladophora glomerata* occurred in alkaline waters elsewhere in the country.

A satisfactory method of monitoring the time and rate of algal invasion was developed during the project. This involved fastening plastic petri dishes to a board suspended in the canals in such a way that they could be detached for laboratory examination.

There were a number of cases of illegal control with copper sulphate, possibly by the farmers themselves, which made the recording of the growth of algae related to environmental conditions frustratingly difficult for the project team. However, it was established that *Cladophora* recruitment can occur throughout the year. During the project *Oedogonium* blooms were confined to only one part of the Breede River System. Control with copper sulphate is easier in this system as the water is naturally slightly acid to neutral, and does not need the pH to be lowered before treatment.

Laboratory culture of *Cladophora* is difficult, but a technique was developed which gave acceptable results. This allowed some laboratory work on basic growth requirements, as well as determining the relationship between copper uptake and environmental variables. This, in turn, enabled specific recommendations to be made regarding the use of copper sulphate in the control of the algae

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The potential biological control agents of Cladophora glomerata that occur in irrigation schemes in South Africa

AR Wood

The filamentous alga Cladophora glomerata is a serious pest in canal systems, occurring in canals serving irrigation schemes as well as other industries such as water boards and power stations. This project investigated the possibility of using pathogens as a method of biological control against the alga, an option which has received little attention elsewhere against this pest.

In the field a wide variety of potentially pathogenic organisms was observed on the alga. However, the organisms of potential interest for biological control were all fungi. Fungi belonging to the groups Chytridiomycetes and Oomycetes were observed, but since these cannot be cultured on artificial media, they were not investigated further. They may, however, be important pathogens, and so a useful component of an integrated control programme. Hyphae of the fungal genus *Pythium* were the most frequently isolated, and were isolated from living, degenerating and dead cells. These were assumed to be pathogenic. Eleven groups of isolates were identified by the zoosporangia produced. These and other isolates were tested in initial pathogenicity tests, and were found able to penetrate partly damaged cells within 24 h of exposure.

This research is being continued in a current project, entitled An Investigation into the use of pathogenic organisms for the biological control of the troublesome alga Cladophora glomerata

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