Novel mycoherbicides for biological control of aquatic weeds such as Water Hyacinth and Water Lettuce©: Patent Disclosure.

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Abstract of the invention

An invention is described about biological control of noxious aquatic weeds. Research in tropical greenhouses revealed the discovery of two fungal pathogens both capable to kill Water Hyacinth and Water Lettuce. These pathogens were developed as 'mycoherbicides' to eradicate invading weeds in tropical and subtropical waterways. Treatments of Water Hyacinth and Water Lettuce led to sudden death, sinking, and early dying. More than 90% of these invading weeds were killed in greenhouse trials.

Summary of the invention

The invention relates to the application of the fungus *Sclerotinia sclerotiorum* as a mycoherbicide to control aquatic weeds such as Water Hyacinth (*Eichhornia crassipes*) and Water Lettuce (*Pistia stratiotes*). *S. sclerotiorum* has been found to be capable to infect and kill Water Hyacinth and Water Lettuce when mycelium of the fungus is applied to the plants in a suitable formulation. *S. sclerotiorum* has never been reported as being pathogenic to Water Hyacinth and Water Lettuce. Accordingly, the biological control method to control aquatic weeds using mycelium of *S. sclerotiorum* is novel.

The invention also relates to the application of the pathogenic fungus *Thanateporus cucumeris* AG 2-2 to control the aquatic weeds Water Hyacinth and Water Lettuce. Mycelium of the anastomosis group (type 2) of this fungus has never been reported as being of use in the biological control of Water Lettuce. Accordingly, the said applications for biological control of aquatic weeds are novel, new and have never existed anywhere in the world. Specified applications of the respective pathogens in combination with the specified aquatic weeds were applied as prototypes of the fungi, named Hyakill™ and Lettkill™, respectively. In greenhouse experiments these prototypes appeared to be very effective in Experiments I-III for the biological control of the specified aquatic weeds. Hyakill™ leads to mottling with Water Hyacinth and yellowing of Water Lettuce and finally to sudden death, early dying of Water Hyacinth, and yellowing of leaves, sinking, early dying of Water Lettuce. Following inoculation by Lettkill™ leads soon to wilting of Water Hyacinth and Water Lettuce plants and finally early dying of these plants.

In conclusion, specified prototypes, based upon mycelium of *Sclerotinia sclerotiorum* and *Thanatephorus cucumeris* AG 2-2, are useful for biological control of

specified aquatic weeds and may accomplish effective biological control. Hyakill™ and Letkill™ based upon prototypes of these pathogens are novel, and unique products.

Background of the invention

Water Hyacinth (*Eichhornia crassipes* (Martius) Solms; family: *Pontederiaceae*) and Water Lettuce (*Pistia stratiotes* L.; family: *Araceae*) are noxious, monocotyledonous aquatic weeds obstructing waterways in tropical and subtropical regions of the world. Water Hyacinth has been called the worst aquatic plant in the world. The weed can be removed by manual control (hand pulling, raking), mechanical removal (using a machine), chemical control (with herbicides such as 2,4-D® and Roundup® (Glyphosate), and biological control with help of insects (weevils). Mechanical removal is very laborious, chemical control using herbicides has disadvantages to the ecosystem, biological control with help of insects is not always very effective.

Mycoherbicides are living fungi that kill living (unwanted) plants (weeds). In the mycoherbicide strategy pathogenic fungi are employed that suppress, and eventually kill the target weed. At present there are no mycoherbicides effectively used in practice to control aquatic weeds. This invention relates to a biological control method by using the mycoherbicide approach.

State of the Art

There are about thirty patents on mycoherbicides of terrestrial, arable weeds. These may be found by linking with help of the internet browser to the Patent Office esp@cenet as to perform quick searches in the worldwide database on patents. Using as keyword: ‘mycoherbicide’, ‘bioherbicide’, ‘biological weed control’, ‘biocontrol’, ‘biological control’ results in about thirty patents. The search term ‘Water Hyacinth’ leads to the interesting mycoherbicide patent on the fungus *Cercospora rodmanii* US4097261, entitled “Method and compositions for controlling Water Hyacinth”. Next pathogenic fungi are listed in a recent synopsis of highly virulent and useful pathogens: *Acremonium zonatum, Alternaria eichhorniae, Cercospora piaropi (=C. rodmanii), Myrothecium roridum, Rhizoctonia solani* (=*Thanatephorus cucumeris*), and *Uromyces eichhorniae.*

Modern research on mycoherbicides to control Water Hyacinth has been focused on two fungal species, namely *Cercospora rodmanii*, *Alternaria eichhornia*. Interestingly, *Cercospora rodmanii* is named in patent US4097261 entitled ‘Method and composition for controlling Water Hyacinth’. Recently, *Rhizoctonia solani* (order Mycelia Sterilia, anamorph is *Thanatephorus cucumeris* Kühn.) has been suggested as a possible candidate for a mycoherbicide to control Water Hyacinth.

Sclerotinia sclerotiorum (Lib.) de Bary is known as a plurivorous plant pathogen, but it has never been observed on Water Hyacinth. Plants susceptible to this pathogen encompass many dicotyledonous plant families, genera and species. Using such an omnivorous pathogen is a novel application of *Sclerotinia sclerotiorum* as a mycoherbicide. The novelty of this application is that a fungal pathogen is employed that is pathogenic to many arable crops such as bean, sunflower, carrot. *S. sclerotiorum* is geographically cosmopolitan and has a broad ecological distribution. The fungus is seldom observed on monocotyledonous, and never observed on aquatic species such as Water Hyacinth and Water Lettuce. Accordingly, our approach is exceptional, because common use in the discipline of mycoherbicides is to isolate and select a pathogen observed on the target weed. *S. sclerotiorum* is being investigated by AgResearch, Lincoln New Zealand as a mycoherbicide candidate for the control of terrestrial, dicotyledonous weeds such as Californian Thistle (*Cirsium arvense*), Giant Buttercup (*Ranunculus acris*), and other weeds in pastures of New Zealand. Grass species in pastures are monocotyledonous species.

Lettkill™ uses mycelium of the pathogenic fungus *Thanatephorus cucumeris* Kühn. (anamorph *Rhizoctonia solani*). It is a very common soilborne pathogen with a great diversity of host plants. Hyphal anastomosis criteria place *R. solani* in taxonomically diffent groups, so-called Anastomosis Groups (AG). In a field survey, it was found to be very destructive on Water Hyacinth. Despite its high virulence and destructive capabilities, *T. cucumeris* has never been investigated as mycoherbicide candidate because of its reputed wide host range. *T. cucumeris* is the sexual state of *R. solani*, called the teleomorph. Infrequently, sexual spores are observed on disease plants. Characteristics of these spores resulted in a name change of *R. solani* into *T. cucumeris*, a *Basidiomycete*. The novelty of this research is that an anastomis group and mating strain of *T. cucumeris* was used (AG 2-2) that has never been reported before for biological control of Water Hyacinth and Water Lettuce. Among the host-plants of AG 2-2 is rice, turf grass, corn, sugar beet.

The pathogenic fungi used in the present invention (*Sclerotinia sclerotiorum* isolated *from Helianthus annuus* L.) and *Thanatephorus cucumeris* AG 2-2 are deposited in the Collection of Biological Farming Systems Group, Wageningen University. The Biological Farming Systems Group offered facilities to make prototypes of mycoherbicides used in the present research. Prototypes and fungal isolates are available for demonstration purpose. Sclerotia of the applied *S. sclerotiorum* isolated from *Helianthus annuus* (sunflower) are stored in a container in the laboratory of the Biological Farming Systems Group. Numerous photographs were made of inoculated Water Hyacinth and Water Lettuce in Experiments I-III with help of a digital camera. These photographs are stored on CD and available upon request.

Mycelium of the fungi was grown in vessels containing 1%-malt extract solution and magnetic stirrers, and subsequent inoculation of Water Hyacinth and Water Lettuce by spraying a mycelium suspension on the leaves. Whilst applying for a patent, we consider the prototype formulations based upon *S. sclerotinia* and *T. cucumeris* as being registered. Accordingly, we use provisional brand names by calling these prototypes Hyakill™, based upon mycelium of *S. sclerotiorum* and Letkill™, based upon mycelium of *T. cucumeris* AG 2-2, respectively. These brand names were given an unofficial TradeMark by making it user names of e-mail addresses on Internet, hyakill@yahoo.com and lettkill@yahoo.com respectively. The inventors used in their communications coding words for the involved pathogenic fungi, such as 'elephant' for *S. sclerotiorum*, and 'giraffe' for *T. cucumeris*. Our method of biological using the mycoherbicide strategy can be considered as novel, non-obvious, and original. Experiments I-III describe the discovery that said aquatic weeds are susceptible to said pathogens. Accordingly, said pathogens can be utilized in practice as mycoherbicides.

Experiments illustrating the invention

Experiment I

Time & Place: Inoculation March 7th and May 15th, 2002, tropical greenhouse, Wageningen University; Biological Farming Systems Group, Wageningen, The Netherlands; average temperature in greenhouse: 22-28 °C. Plants were placed in a plastic tank filled with water, and addition of a NPK-fertilizer to the water.

Six Water Hyacinth (*E. crassipes*) plants were obtained from a local shop of garden plants, 'de oude tol'. Two plants served as *non-inoculated* control, one plant was inoculated March 7th, 2002. Three plants were inoculated (one plant *non-inoculated*) April 16th, 2002 by mycelium suspension of *Sclerotinia sclerotiorum*. *S. sclerotiorum* was isolated from dark-brown sclerotia in the stem of a dead sunflower plant (*Helianthus annuus* L.) with stem rot disease. Mycelium of the fungus was grown in a fermentation unit consisting of magnetic stirrer equipment, explained in: http://www.dpw.wageningen-ur.nl/biob/organis/meindert.htm. Formulations used in current and subsequent experiments: Formulation[a]: no additives to mycelium suspension, Formulation[b]: cellulose and paraffin oil added to mycelium suspension, Formulation[c]: cream emulsion added to mycelium suspension.

A pilot study (inventive step #1) comprised primary greenhouse testing by spraying 2 ml of a mycelium suspension of *S. sclerotiorum* by a lab syringe on leaves of Water Hyacinth. A few days following inoculation, big lesions on leaves appeared. Plants had big lesions (up to 2 cm^2 consisting of numerous small lesions of 1 mm^2 on leaf blades and petioles i.e. apparent mottling one week after inoculation, soon followed by turning brown of leaf tips.

Table I: Numbers of Water Hyacinth (*Eichhornia crassipes*) plants with Sudden death and Early dying due to inoculation by mycelium of *Sclerotinia sclerotiorum* and days of observation after inoculation.

Objects	Sudden death	days;	Early dying	days;	Total number
non-inoculated	0		0		2
S. sclerotiorum	1	30-60	3	>60	4

'Sudden death' (Table I) refers to a change from healthy into affected by serious crown rot followed by degradation of the stem and rotting of leaf blades and petioles, wilting of leaves and roots, crown rot and subsequent losing of the floating capability of the plant. One plant of Water Hyacinth (Table I) had crown rot, split, rotted, and sank to the bottom of the container on July 15th, 2002. This started about 30 days after the first signs of stunted growth of leaves and roots. Finally it was leading to 'sudden death' (Formulation[c] was used). Stunted growth of roots and leaves, and crown rot (disintegration in the heart of the Water Hyacinth rosette) was observed. Water Hyacinth has leaf petioles and blades acting as air cushions that keep the plant floating. Disease can transform the plant into a soft, slimy, water-soaked mass.

'Early dying' (Table I) is defined as dying within two months following inoculation. It is due to an early senescence of leaves and roots leading to dying prematurely, far before expected average life time age. Observed symptoms: two plants of Water Hyacinth (Table I) had one month after inoculation some slime in the leaf rosette during a few days. This symptom is considered as due to infection by *S. sclerotiorum*. Two plants had a chlorotic period of two weeks with necrotic areas around the leaf veins, but they continued growing slowly. In this Experiment dying offspring was observed: new plants produced on stolons had blight symptoms and died as well. Anthracnose was sometimes observed. Dry lesions with an obvious separation between healthy and diseased tissue were observed.

Conclusion: *S. sclerotiorum* can kill Water Hyacinth. The observation that Water Hyacinth is susceptible to *S. sclerotiorum* is a discovery that has never been reported before. Accordingly, *S. sclerotiorum* is a much-promising candidate as mycoherbicide.

Experiment II

Time & Place: Tropical Greenhouse Deventer, Larenstein College, Brinkgeverweg 69, 7413 AB Deventer, The Netherlands. Inoculation date: May 30th, 2002. Average temperature in greenhouse: >24°C Aquatic weeds: Water Hyacinth, Water Lettuce plants (3 cm diameter, 4-5 leaves stock plants in greenhouse Larenstein, Deventer). Fungal pathogens: *S. sclerotiorum* (Helianthus annuus isolate), *T. cucumeris* AG 2-2 TC, collection Biological Farming Systems Group, Wageningen University). On the bottom of the container, a thin layer of potting ground was added.

Inventive step #2 conveys a repeat and testing *S. sclerotiorum* on much more plants, involvement of one more aquatic weed (Water Lettuce, *P. stratiotes*) and one more pathogenic fungus (*T. cucumeris*). 32 Water Hyacinth plants and 4 Water Lettuce plants were inoculated. The pathogenicity of *S. sclerotiorum* is compared with *T. cucumeris* using two aquatic weed species (Water Hyacinth and Water Lettuce) in order to have reference values for pathogen-aquatic weed combinations.

Following inoculation of Water Hyacinth by mycelium of *S. sclerotiorum*, four plants (i.e. 22%) died due to sudden death. Many plants (67% after inoculation by *S. sclerotiorum* and 71% by *T. cucumeris*) had early dying (Table IIa). Two Water Hyacinth plants inoculated with *S. sclerotiorum* turned yellow in ten days time after inoculation by *S. sclerotiorum*. Inoculation of Water Hyacinth by mycelium of *T. cucumeris* led to lethal yellowing and crown rot. Internal air spaces in leaves and hairs on leaves of Water Lettuce keep the plant floating. Disease may lead to sinking: plant remains sink all of a sudden to the bottom of the container. Disease symptoms caused by *T. cucumeris* consist of irregularly shaped necrotic spots, and broad lesions. Brown necrotic areas were usually surrounded by noticeable, thin, water-soaked margins of darker brown color than the rest of the lesion. Observed symptoms on Water Lettuce included: discoloration, yellowing of leaves, stunted growth of new leaves (in sprout) and roots, blight, brown and stripy lesions were visible.

Table IIa: Numbers of Water Hyacinth (*Eichhornia crassipes*) plants with Sudden death and Early dying due to inoculation by mycelium of *Sclerotinia sclerotiorum* and *Thanatephorus cucumeris* AG 2-2 and days of observation after inoculation.

Object	Sudden death days;	Early dying days;	Total number

non-inoculated	0		0		5
S. sclerotiorum	4 (22%)	>10	12 (67%)	>50	18
T. cucumeris AG 2-2	0		10 (71%)	>70	14

Results of inoculation of Water Lettuce show sinking and early dying (Table IIb). Apparently, both fungi, *S. sclerotiorum* and *T. cucumeris* AG 2-2), can infect and cause disease with Water Lettuce.

Table IIb: Numbers of Water Lettuce (*Pistia stratiotes*) plants with Sinking after inoculation by mycelium of *Sclerotinia sclerotiorum* and *Thanatephorus cucumeris* AG 2-2, and days of observation after inoculation.

Object	Sinking	days;	Early dying	days;	Total number
non-inoculated	0		0		5
S. sclerotiorum	0		1	>30	2
T. cucumeris AG 2-2	2	7	0		2

Experiment III

Time & Place: July 18th, 2002. Tropical greenhouse, Deventer, The Netherlands.
Inventive step #3 conveys a repeat of Experiment II, and involvement of much larger Water Lettuce plants (leaves were 30 cm length, crown diameter was 10 cm) than in Experiment II.

Ten plants of Water Hyacinth and fourteen plants of Water Lettuce were inoculated. Fluffy mycelium spots were sometimes visible (“spots of white mold”) on leaf blades and petioles of Water Hyacinth plants inoculated by *S. sclerotiorum* (especially with Formulation[c]). Water Hyacinth plants had sudden death and early dying due to inoculation by *S. sclerotiorum* and *T. cucumeris* (Table IIIa).

Table IIIa: Numbers of Water Hyacinth (*Eichhornia crassipes*) plants with Sudden death and Early dying due to inoculation by mycelium of *Sclerotinia sclerotiorum* and *Thanatephorus cucumeris* AG 2-2, and days of observation after inoculation

Object	Sudden death	days;	Early dying	days;	Total number
non-inoculated	0		0		7
S. sclerotiorum	0		2	>30	6
T. cucumeris AG 2-2	0		4	>30	4

Two Water Lettuce had a sudden sunk in two weeks after inoculation, and early dying due to inoculation by *S. sclerotiorum*, and *T. cucumeris* (Table IIIb).

Table IIIb: Number of Water Lettuce (*Pistia stratiotes*) with Sinking, and Early dying after inoculation by mycelium of *Sclerotinia sclerotiorum* and *Thanatephorus cucumeris* AG 2-2, and days of observation after inoculation.

Object	Sinking	days;	Early dying	days;	Total number
non-inoculated	0		0		52
S. sclerotiorum	2	20	0	>30	2
T. cucumeris AG 2-2	2 (17%)	20	9 (75%)	>40	12

Leaves of four big Water Lettuce plants inoculated by mycelium of *S. sclerotiorum* turned cracky yellow two days after inoculation, followed by extreme wilting of the whole plant. Two Water Lettuce plants inoculated by mycelium of *T. cucumeris* had mild yellow leaves, while two Water Lettuce plants inoculated by *S. sclerotiorum* showed cracky yellow of leaves and extreme wilting. These plants died soon.

Generally, terrestrial plants with disease due to *S. sclerotiorum* develop many sclerotia in dead stem tissue. However, such sclerotia have never been developed in inoculated Water Hyacinth in any Experiment.

Utility

The mycoherbicides described in this invention are based upon plant pathogens that may cause risks to agricultural crops. Ways of avoiding risks of said mycoherbicides to non-target plants is best investigated in collaboration with a mycological and phytopathological department of a national university, agricultural-environmental, governmental institute.

An alternative way of risk reduction of the biological control method using *S. sclerotiorum* may be the use of patent US5538890, entitled: “self-delimiting mutants, bioherbicidal compositions thereof, method for preparing thererof, and method of using thereof for weed control”. A mycological way of risk reduction would be to select strains of *S. sclerotiorum* and *T. cucumeris*) that are hardly pathogenic to arable crops. This can be developed in collaboration with a national university, agricultural-environmental, governmental institute.

In plant protection, introducing of a foreign pathogen needs quarantine measures. Accordingly, the pathogens for Hyakill™ and Lettkill™ (*Sclerotinia sclerotiorum*, *Thanatephorus cucumeris* AG 2-2, respectively) are to be isolated in the region, nation, country, earth continent of application (e.g. from a locally occurring crop disease in sunflower, bean, soybean, cowpea, or available from a plant disease centre, university). Production, manufacturing, entrepreneurship should preferably be in the country of biological control practice for safety reasons and to avoid quarantine measures. In conclusion, it is recommended to perform field trials in practice in strong collaboration with a national university, agricultural-environmental, governmental institute.

The experiments and embodiments described herein are for illustrative purposes only. It will be apparent that a number of variations in the foregoing invention may be practiced without departing from the scope and spirit of the invention.

Claims

1. Two mycoherbicide concentrates[a,b] comprising a mycelium suspension based upon the fungal pathogen *Sclerotinia sclerotiorum* (Lib.) de Bary[a], and a mycoherbicide concentrate comprising a mycelium suspension based upon the fungal pathogen *Thanatephorus cucumeris* (Frank) Donk; anastomosis group AG 2-2 (anamorph *Rhizoctonia solani* Kühn.)[b], to prepare formulations effective to control Water Hyacinth (*Eichhornia crassipes* (Martius) Solms) and Water Lettuce (*Pistia stratiotes* L.).
2. Mycoherbicide concentrates[a,b] for the preparation of compositions effective to control Water Hyacinth and Water Lettuce in claim 1 said concentrates contain any slurry of the microorganisms *Sclerotinia sclerotiorum* and *Thanatephorus cucumeris* AG 2-2.
3. *Sclerotinia sclerotiorum* cominbed with *Thanatephorus cucumeris* as mycoherbicides in biological control of Water Hyacinth and Water Lettuce.
4. The methods used for cultivating said mycoherbicides[a,b] in claim 1 are not particularly critical provided such methods result in an effective composition with an adequate quantity of viable mycelium to achieve the desired effect.
5. Said mycoherbicides[a,b] in claim 1 are to be embodied in an environmentally acceptable carrier.
6. *Sclerotinia sclerotiorum* for the preparation of a mycoherbicide[a] in claim 1 is not to be restricted to a *Helianthus annuum* L. isolate only.
7. *Thanatephorus cucumeris* for the preparation of mycoherbicide[b] in claim 1 is not to be restricted to anastomosis group AG 2-2 only and is to be isolated in the target region, country, continent of biological control application.
8. Said fungi for the preparation of mycoherbicides[a,b] in claim 1 and claim 2 are to be isolated in the target region, country, continent of biological cpmtrol application.

Stakeholders

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Advice on the claims were given by dr Peter Lüth (PROPHYTA GmbH, Inselstraße 12, 23999 Malchow, Germany, plueth@prophyta.com Sir John Loyd, CropCare Ltd, Nelson, New Zealand olympus46@xtra.co.nz Dr Graeme W. Bourdot (AgResearch Ltd, Lincoln, New Zealand gave general advice about patenting. Advice to patent our invention was given by 'BioPartner', Center Wageningen www.biopartnerwageningen.nl

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Advice on business and patent matters was given by dr Siebe van de Geijn (head, Wageningen University and Research), mr Lidwien Dubois (Advocate Intellectual Property; lidwien.dubois@wur.nl, dr Dion E.A. Florack (PRI, Wageningen), and mr Margriet de Kleter. Prof. dr Raghavan Charudattan, Plant Pathology, University of Florida, Gainesville, USA. Dr Niels Holst, Denmark gave advice. Martijn Staats (Phytopathology, Wageningen University) provided a *Botrytis cinera* culture for additional testing. Prof. Geert Smant and Prof. Jan van Bezooijen (Nematology, Wageningen University) determined bacteriofage nematodes in one container with inoculated Water Hyacinth. Mr Dik van Harte (Octrooi-Adviseur), Netherlands Industrial Property Office, Marketing & Public Relations, Patentlaan 2, Rijswijk, Netherlands. Mr Arthur Meekel, Nederlandsch Octrooibureau, Den Haag, advised about patenting meekel@octrooibureau.nl
We discussed our invention with dr Arnold Pieterse (Royal Tropical Institute, Amsterdam, Netherlands), dr Piet C. Scheepens, Wolter van der Zweerde MSc (Plant Research International, Wageningen, the Netherlands), Prof. Bruce Auld (University of Sydney, Australia) dr Mic Julien (CSRO, Brisbane, Australia), dr Fen Beed, Institute of Tropical Agriculture, Research to Nourish Africa, Cotonou Station, Benin, West Africa; dr Paul Deprez (Nordevco Associates Ltd., Winnipeg, Canada). Additonsl correspondence over e-mail with Robert C. Anderson MSc (Pacific Islands Ecosystems Research Center, Honolulu, Hawaii), dr Maurizio Vurro (IBG Newsletter, Italy), Prof. Dr Pierre de Wit (Phytopathology, WUR).

Appendix

Accompanying photos:

left: CONTROL april 8 2002 right: TREATED

Eichhornia bioherbicide. Inoc.date 03/03/02 by DeJong-DeVoogd


5/06