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Full Length Research Paper

Production and oil-emulsion formulation of *Cadophora malorum* and *Alternaria jacinthicola*, two biocontrol agents against Water Hyacinth (*Eichhornia crassipes*)

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Cadophora malorum isolate Mln715 and Alternaria jacinthicola strain MUCL 53159 are under development as biocontrol agents against Water Hyacinth (Eichhornia crassipes) in Mali. Production of spores of these agents on locally available substrates (Water Hyacinth, powdered paddy rice chaff, wheat semolina) was assessed with a view to mass production. The C. malorum isolate sporulated best on Water Hyacinth (4.08 × 107 spores ml-1), followed by wheat (1.06 ×107 spores ml-1), whereas A. jacinthicola produced more spores on paddy rice chaff and wheat (0.24 x 107 spores ml-1). The severity of the damage caused by each pathogen was evaluated in the greenhouse and in the field. Under both greenhouse and field conditions, the biocontrol efficacy of the fungal isolates was improved with (unrefined) Carapa procera (L.) oil or (refined) palm oil, supplemented with soybean lecithin and Tween 20. When such a formulation was used, the incubation time was 4 to 5 days in the greenhouse and 7 to 9 days on the field, and the damage severity (DS) recorded 6 weeks after treatment varied from 87.02 to 93. 13% in the greenhouse and from 59.11 to 63.00% in the field. For unformulated C. malorum and A. jacinthicola respectively, the incubation times were longer and the DS values were only 22.11 and 29. 05% in the greenhouse and 12.05 and 15.15% on the field. Our results highlight good substrates for mass production of these mycoherbicides and demonstrate the ability of vegetable oil formulations to improve their efficacy.

Key words: *Alternaria jacinthicola*, biocontrol, *Cadophora malorum,* oil formulation, Water Hyacinth.

INTRODUCTION

Water Hyacinth (*Eichhornia crassipes* (Mart.) Solms-Laubach) originating from the Amazon Basin in South America is a free-floating aquatic weed that is found worldwide. *Eichhornia crassipes* belonging to the family *Pontederiaceae* is considered the world's worst aquatic weed (Lata and Dubey, 2010). It causes global annual losses in excess of US$ 100 million for hydro-electricity plants, irrigation schemes, fisheries, riparian communities, and activities relying on water transit (Adebayo and Uyi, 2010; Gopal, 1987).

Since 1980, rice and irrigated orchards have been the most important sources of food and income for farmers in Mali, especially in the Koulikoro, Segou, and Niono regions along the Niger River (Dagno, 2006). Many diseases, insects, and weeds, however, limit the stability of rice and fruit production. In particular, infestation of the Niger River by Water Hyacinth has increased drastically. This aquatic weed decreases the water flow by clogging dams and irrigation work.

Herbicides (Paraquat, Diquat, Glyphosate, Amitrole, 2, 4-D acid or amine) have been widely used to manage Water Hyacinth in water bodies (Land Protection, 2001), but their residual toxicity and deleterious effects have

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prompted the search for eco-friendly alternatives for controlling the weed (Gupta et al., 2002).

An alternative envisaged is biological weed control based on the use of natural microorganisms (Charudattan, 2005). The classical approach involving the use of exotic plant pathogens was developed in the early 1970s (Watson, 1991). *Puccinia chondrilla*, for example, has been used effectively to control skeleton weed (*Chondrilla juncea*) in Australia and the U.S.A (Yang et al., 2000). Another approach is to use an endemic (native) pathogen to control its host weed, by delivering a massive dose of the pathogen during susceptible stages of weed growth (Charudattan, 1991).

Over the last decade, much research has been devoted worldwide to the development of new mycoherbicides. In 2006 to 2007, our team conducted a survey of fungal species present on Water Hyacinth in Mali. Two isolates emerged as promising potential biocontrol agents against this weed: *Cadophora malorum* (Syn. *Phialophora malorum*, *Sporotrichum malorum*) isolate Mln715 and *Alternaria jacinthicola* strain MUCL 53159, representing a new species (Dagno et al., 2010). In keeping with the conclusion of Boyette et al. (2007) that water activity and temperature are the two most important environmental factors influencing mycoherbicide efficacy, the germination potential and mycelial growth of these two strains show a clear temperature optimum (near 25°C) and are negatively affected by low water activity (Dagno et al., 2010). Such influences make it important to consider with care the formulation of biocontrol agents, recognised as an important determinant of their efficacy (Greaves et al., 1998; Auld, 1993). Furthermore, having a cheap and locally available substrate on which to mass-produce inocula can also be important (Siddiqui et al., 2008).

The objective of the present work was to determine, under greenhouse and field conditions, the effects of oil-water emulsions (prepared with unrefined *Carapa procera* (L) oil or refined palm oil and amended with soybean lecithin and Tween 20) on the germination of *C. malorum* (isolate Mln715) and *A. jacinthicola* (strain MUCL 53159) spores and on the severity of the damage they cause to Water Hyacinth. We have additionally tested three locally available plant materials as substrates for economically viable mass production of *C. malorum* and *A. jacinthicola* strain MUCL 53159.

MATERIALS AND METHODS

Fungus and plant preparation

C. malorum isolate Mln715 is stored at the Plant Pathology Unit, Gembloux Agro-Bio Tech-University of Liege, Belgium (at -70°C, in tubes containing 25% glycerol). *A. jacinthicola* strain MUCL 53159 is stored in the Industrial Fungus and Yeast Collection (BCCM/MUCL) in Belgium. The initial conidial inocula used in the present experiments were taken from Petri-dish cultures on potato dextrose agar (PDA, Merck, Darmstadt, Germany), preserved at 4°C for not more than 6 months, and then subcultured at 25°C on different culture media before use. Healthy Water Hyacinth plants were collected and sampled from a naturally infested area of the Niger River in Mali and grown in a sterilized greenhouse.

Evaluation of potential substrates for mass production of fungal strains

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

Conidial viability

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

Evaluation of the efficacy of vegetable oil emulsion formulations under greenhouse conditions

Vegetable oil emulsions were prepared with 35% v:v refined palm oil or unrefined *Carapa procera* (L.) oil amended with 15% soybean lecithin (used as an emulsifying agent) and 5% Tween 20 (used as a formulation adjuvant) (all concentrations mentioned are final concentrations). On the one hand, Tween 20 was diluted in sterile distilled water containing the spores (at a concentration calculated to yield a concentration of $5x10^6$ spores ml^{-1} in the final oil-water emulsion). On the other hand, preheated soybean lecithin was added to the vegetable oil phase and the resulting mixture was homogenised with a blender. The oil and aqueous phases were then combined and vigorously homogenized with an electric mixer.

Each oil-emulsion formulation was sprayed onto hyacinth plants at the 3- to 5-leaf stage. Three types of controls were also included: “Control I” plants were sprayed with unformulated fungus (50 ml of the aqueous phase described above, containing 5×10^6 spores ml^{-1}), “Control II” plants with sterile distilled water without fungus, and “Control III” plants with vegetable oil only. Treated plants were incubated under the same conditions as described above. Three replicates were prepared for each treatment and arranged in a

complete randomized design. After six weeks, the plants were rated for disease symptoms including leaf spots, leaf lesions, and leaf death.

The impact of the pathogens was determined by counting the number of leaf spots and leaf lesions per leaf and by assessing the damage severity (DS). The DS was derived according to Freeman and Charudattan (1984), from ratings on a scale of 0 to 9, where 0 = healthy, and 9 = 100% damage. Scores for individual leaves were summed and averaged, and the mean score converted to a percentage for a whole plant. The experiment was repeated three times.

Field trials

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

Data analysis

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

RESULTS

Conidial yield and viability

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

The viability of the *C. malorum* and *A. jacinthicola* conidia produced on these plant substrates was evaluated on Water Hyacinth leaves. Whatever the pathogen, germ tubes were clearly visible on leaves (at 100× magnification) 4 h after inoculation. The mycelium was found to colonize the leaf surface and branches before entering through stomata or intercellular spaces and colonizing the whole leaf 8 h after inoculation.

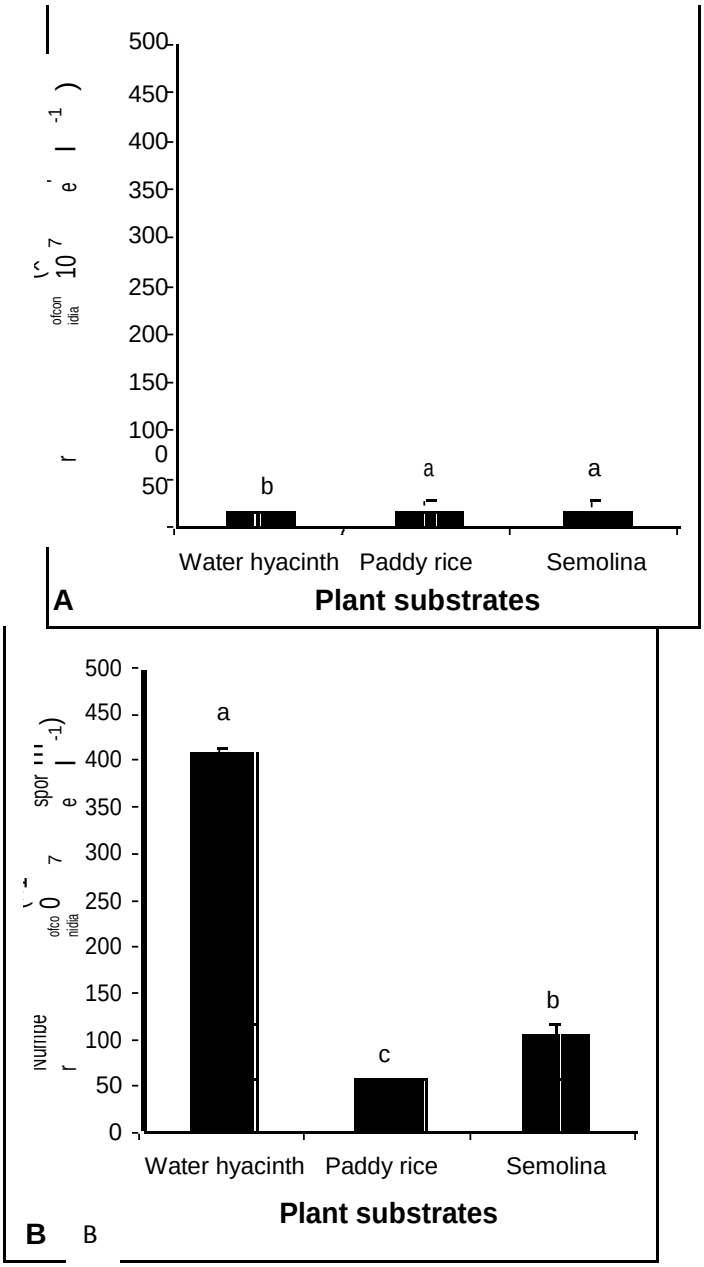

Figure 1. Production of biocontrol agents on plant substrates 4 weeks after incubation. (A) *Alternaria jacinthicola* (strain MUCL 53159) ; (B) *Cadophora malorum* (isolate Mln715). Treatments with the same letters are not significantly different according to Duncan tests (*P* < 0.05). Bars represent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

Evaluation of vegetable oil emulsion efficacy under greenhouse conditions

A preliminary small-scale greenhouse trial was conducted at the Plant Pathology Unit, Gembloux Agro-Bio Tech to evaluate the biocontrol efficacy of both fungal isolates against Water Hyacinth. The incubation or latency period (time elapsed between exposure to a pathogen and the appearance of the first symptoms); the number of

Table 1. Efficacy of variously formulated biocontrol agents against Water Hyacinth. DS values were recorded 6 weeks after treatment.

Treatments	Greenhouse conditions	Field conditions
Treatment 1	93.13±0.42	63.00±3.60
Treatment 2	87.02±1.02	59.11±2.75
Treatment 3	90.35±0.50	63.02±3.10
Treatment 4	87.05±1.09	62.21±2.32
Control I1	22.11±1.32	12.05±5.20
Control I2	29.05±1.52	15.15±4.80
	Damage severity DS (%)	
Control II	0.00±0.00	5.20±5.30
Control III1	5.25±0.00	9.20±3.85
Control III2	6.10±0.00	10.18±3.25

DS: Damage severity, Treatment 1: *Cadophora malorum* + palm oil, Treatment 2: *Cadophora malorum* + *Carapa procera* (L) oil, Treatment 3: *Alternaria jacinthicola* + palm oil, Treatment 4: *Alternaria jacinthicola* + *Carapa procera* oil, Control I1: *Cadophora malorum* unformulated, Control I2: *Alternaria jacinthicola* unformulated, Control II: distilled water, Control III1: palm oil, Control III2: *Carapa procera* (L) oil. Greenhouse conditions (relative humidity = 55 ± 5; temperature = 25°C; 16 h photoperiod).

diseased leaves, and the leaf area covered by disease were estimated. In this experiment, all oil-emulsion-treated samples (including the pathogen-free controls) showed slight leaf necrosis from Day 1 post-inoculation onward, but the onset of symptoms due to either pathogen was nevertheless clearly distinguishable.

With both fungal species, the latency period was 4 days for the pathogen formulated in palm oil and 5 days for the pathogen formulated in *C. procera* (L) oil.

Plants pulverized with unformulated *C. malorum* or *A. jacinthicola* (Control I) showed a 9-day latency.

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

Field trials

Field trials were conducted in naturally infested parts of the Niger River in the District of Bamako in Mali. In these trials, the incubation period for oil-formulated *C. malorum* was 7 (palm oil) or 8 days (*C. procera* oil), as opposed to 12 days in the case of the unformulated pathogen. The incubation period for *A. jacinthicola* was 9 days (*C. procera* oil), 8 days (palm oil), or 12 days (unformulated). Whatever the oil, the oil-formulated fungi showed very similar efficacy (DS = 59.11 to 63.00%), the only significant difference being a slightly lower DS for *C. malorum* in *C. procera* oil. Plants treated with oil alone again showed slight necrosis from day 1 post-pulverisation onward, and in the distilled water controls, slight symptoms appeared after 13 days (DS = 5.20% after 6 weeks). Over the test period (February and March 2009), the average temperature recorded at 2:00 PM was 35°C and the average temperature recorded at midnight was 15°C. The average RH was 55% by day and 85% by night.

DISCUSSION

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

Figure 2. Healthy Water Hyacinth plant (A); leaf blight symptoms caused by *Cadophora malorum* (in formulation with palm oil) (B) and by *Alternaria jacinthicola* (in formulation with palm oil) (C) 6 weeks after inoculation of greenhouse-grown Water Hyacinth plants.

2005; Vincent, 2001).

Here, we have focused on two fungal pathogens isolated from diseased Water Hyacinth plants in Mali, with a view to developing them as effective biocontrol agents. We have notably considered the problem of local mass production. Several plant substrates such as wheat straw; chickpea flour; rice straw, sorghum, and rice have been used to mass produce BCAs (Siddiqui et al., 2008; Abbas et al., 2000), but the use of Water Hyacinth and paddy rice chaff as substrates for mass production of *C. malorum* and *Alternaria* species has not been reported previously. In our tests, the former substrate proved best for producing *C. malorum* and the latter for producing *A. jacinthicola*. These substrates should be obtainable locally at low cost, as Water Hyacinth is an abundant weed and rice chaff, an industrial waste.

It should also be relatively simple to use them in developing countries where Water Hyacinth is considered an important threat and where fermentation systems for inoculum production are lacking. In our case, all three tested substrates yielded spores that germinated well on Water Hyacinth.

We have also considered the formulation of these fungi. On the one hand, an adequate formulation can contribute to a uniform distribution of inoculum on the plant surface, essential to creating epidemics (Boyette et al., 2007; Bailey et al., 2004). On the other hand, its use may help to solve a major problem inherent in using leaf fungi for weed control: the fact that such fungi require optimal relative humidity for germination, in order to infect the weed (Shabana, 2005). In relation to this problem, El-Morsy (2006) and Kadir et al. (2000) reported that oil emulsion formulations may reduce dew requirements in field application.

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

Despite the improvements recorded with our oil formulations, our *C. malorum* and *A. jacinthicola* strains proved less effective against Water Hyacinth on the field than in the greenhouse. Similar observations have been made with formulations of *Beauveria bassiana* and *Metarhizium anisopliae* (Bandani and Esmailpour, 2006; (Alves et al., 1998). Such results may be due to factors such as intense solar irradiation (decreasing BCA viability in the field), major variations in temperature and RH between night and noon (in our case, 15°C and 55% RH at midnight vs. 35°C and 85% RH at 2:00 PM), or other microenvironmental factors.

There is thus room for further improvement of the biocontrol efficacy of our strains. A simple solution might

be to increase the concentration of the inoculum, so as to offset any viability-diminishing effects of environmental factors. Boyette et al. (2007) achieved higher efficacy of *Conophytum truncatum* against hemp sesbania in field trials with 1×10^7 spore ml^{-1}. Another approach might be to change the timing of *C. malorum* or *A. jacinthicola* application. Our field assays were performed from February to March, a dry season in Mali. The period from June to October, characterised by rainfall, slighter temperature variations, and good growth of Water Hyacinth, might be better.

Further improvements to the formulation might also be considered. Incorporation of lignin or other UV-protectants into the formulated product, in combination with skimmed milk or another carbon source, may protect a fungal isolate against desiccation and harmful effects of UV light and thus prolong its persistence. It has been demonstrated, for instance, that formulating *Beauveria bassiana* spores with lignin can significantly increase their survival under solar radiation (Leland et al., 2005).

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

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