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Development of Bioherbicides for Control of Barnyard Grass in China

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ABSTRACT

Research progress on bioherbicides against barnyard grass in rice fields was reviewed with a focus on Chinese perspective. In China, barnyard grass is one of the most problematic weeds in paddy rice fields. Several fungal biocontrol agents have been studied extensively and the most promising candidates explored for commercial development, including strain selection and improvement, inoculum mass production, formulation, efficacy trials under various conditions, synergy with chemical herbicides, and safety to crops. Overall, mass production and formulation technologies have proved to be the major stumbling blocks that hinder bioherbicide development. Strategies are discussed to overcome the challenges and facilitate the development of selected fungal agents into commercial bioherbicide products.

Keywords: biocontrol, mycoherbicide, pathogenic fungi, phytotoxin

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INTRODUCTION

In China, barnyard grass (*Echinochloa crus-galli* (L.) P. Beauv.) is one of the most serious weeds found in rice fields (Huang *et al*. 2004). It also causes severe yield losses in legume, potato, cotton and other crops (Wu *et al*. 2006). It is widely distributed in different regions, is highly resistant to environmental stresses and extremely difficult to combat with conventional strategies (Zheng *et al*. 2008). The main means of control has been manual weeding and/or use of synthetic herbicides. Manual weeding is time-consuming and laborious. Large-scale use of herbicides has caused environmental concerns worldwide, including water and soil pollution, disturbance of ecological balance and biological diversity in rural areas, increase in herbicide resistance, thereby making the management of certain weed species a great challenge. Due to repeated use and/or inappropriate class rotation, certain herbicides such as quinclorac, butachlor, and thiobencarb, have encountered serious resistance or tolerance issues with barnyard grass populations in China (Wu *et al*. 2006, 2007; Lu *et al*. 2008).

Microbial bioherbicides could be a new mode of action that may have the potential to replace or supplement some of the current chemical herbicides for effective control of barnyard grass. Unlike chemical herbicides, many microbial bioherbicides, in particular those consisting of a fungus or fungal metabolites (biorationals), tend to have very narrow weed spectrum and more benign impact on the environment. Because some bioherbicides may be compatible with organic rice farming systems, and the potential is appealing. Due to possible advantages of bioherbicides, including environmentally friendly nature, non-target safety, different modes of action for herbicide-resistant weeds, and potential diversity/abundance, many researchers have developed interest of weed biocontrol in recent years (Guo and Kong 2005).

Substantial progress has been made in the research of biological control of paddy barnyard grass. Since 1984, scientists in Portugal, United Kingdom, Germany, the Netherlands, India, South Korea, Japan, Malaysia, Canada and Philippines (International Rice Research Institute) have reported a variety of fungal pathogens with potential for biocontrol of barnyard grass. Some of the promising candidates include *Helminthosporium* sp. (Huang *et al*. 2005), *Cochliobolus lunatus* Nelson & Haasis (Scheepens 1987; Duong *et al*. 1999), *Colletotrichum graminicola* (Ces.) G.W. Wils. (Yang *et al*. 2000), *Drechslera monoceras* (Drechsler) Subramanian & B.L. Jain (Watanabe *et al*. 2001; Hirase *et al*. 2004), and *Exserohilum monocerus* (Drechsler) Leonard & Suggs (Zhang *et al*. 1996; Zhang and Watson 1997; Tsukamoto *et al*. 1999; Kadir *et al*. 2008).

China is one of the earliest countries to develop and commercialize microbial bioherbicides on a large scale. The bioherbicide LUBAO was used widely in China to control

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parasitic dodder (*Cuscuta* sp.) in soybean fields. The active ingredient in LUBAO was a strain of *Colletotrichum gloeosporioides* (Penz.) Sacc. discovered in 1963, and by the late 1970s this bioherbicide was being applied to 670,000 ha of soybean fields in ten provinces. On average, LUBAO provided about 85% control of dodder. The research of bioherbicides for control of barnyard grass is still at early stages in China, and the pace of development and commercialization has been slow due largely to inconsistent funding support. Several fungal pathogens have been studied extensively, including strain selection, formulation, mass production, weed control efficacy under varying conditions, synergy with chemical herbicides, and non-target safety, but some technological barriers including inoculum mass production and formulation have been major stumbling blocks hindering the development beyond the research stages. In the following, discussions will be centered around research progress on biocontrol of barnyard grass in China and outlining strategies to overcome the challenges and facilitate the development of selected fungal agents into commercial bioherbicide products.

DISCOVERY OF POTENTIAL BIOHERBICIDE CANDIDATES

Helminthosporium monoceras Drechs. was the first fungal pathogen reported in China with potential for biocontrol of barnyard. In addition, *E. monoceras*, *Curvularia lunata* (Wakker) Boedijn, *Alternaria alternata* (Fr) Keissler, and *D. monoceras* were also studied extensively. Unfortunately many of these projects were not continued due to a lack of research funding.

Chen and Ni (2001) obtained eight *E. monoceras* (sym. *H. monoceras*) isolates from diseased barnyard grass over several provinces in China. This fungus has also been reported by others as highly virulent pathogen on barnyard grass but safe to rice plants (Aldfidge and Turner 1970; Zhang *et al.* 1996; Caunter 1997; Zhang and Watson 1997; Tsukamoto 1999). The biological and epidemiological characteristics of *E. monoceras*, its host range, and interaction with chemical herbicides for control of barnyard grass have been studied extensively (Chen and Ni 2001; Chen *et al.* 2004, 2005; Yang *et al.* 2005, 2007).

Helminthosporium gramineum Rabenh f. sp. *echinochloae* (HGE) was isolated from infected barnyard grass and considered as another potential biocontrol candidate based on epidemiological traits and crop safety. HGE had higher conidiation yields compared to *E. monoceras* (Huang *et al.* 2005), and a mass production technique using barnyard grass stems and leaves as the raw fermentation substrate was successfully established (Huang *et al.* 2004). This mass-production technology has been granted a patent in China. HGE showed promise when used for control of barnyard grass in rice fields, with 65% weed control after a single application. However, when tank mixed with a 25% label dose of the herbicide quinclorac, the efficacy for barnyard grass control was greater than 90%. When mixed with 10% label doses of quinclorac and bensulfuron, both barnyard grass and sedge (*Cyperus difformis* L.) were controlled with over 90% efficacy. Consequently, the use of the chemical herbicides was reduced by more than 75% in comparison to the label rates (Yu *et al.* 2005).

IMPROVEMENT OF FUNGAL BIOCONTROL AGENTS

Based on fungal biology, ecology, epidemiology, virulence, and host range of the agents identified, *Exserohilum*, *Helminthosporium* and *Drechslera* species appeared most promising for biocontrol of barnyard grass. In reality, it was becoming increasingly difficult to find novel agents against barnyard grass from the natural or cropping environment. As mentioned earlier, obtained isolates of these species often were not perfect candidates for development into bioherbicide products due mostly to poor conidiation and difficulties in formulation. Therefore, technological improvements became essential when going forward.

Using a combination of physical, chemical and modern biotechnological techniques, Huang *et al.* (2004) improved sporulation of a *D. monoceras* isolate. A high-conidiation strain I262 (30% higher than the wild type) was obtained by treating the parental strain with 0.2 mg/ml alkylating agent nitrosoguanidine for 20 min and by screening resulting progenies for greater conidiation. About another 15% improvement in sporulation was achieved later by treating conidial suspensions of the strain I262 with Cobalt 60 gamma-ray.

HGE was also mutagenized with UV to generate variants with higher virulence and biological control ability against *Echinochloa* species (Zhang *et al.* 2007, 2008), and the mutant M1 was found to be suitable for industrial liquid fermentation. Other biological changes also occurred with the M1 mutant when compared to the original HGE isolate, including a change in mycelial color from dark green to white gray, more rapid growth of fungal colony on agar media, and production of metabolites that were more suppressive to the roots and shoots of barnyard grass than the original HGE. After many cultural transfers on artificial media, M1 showed genetic stability based on random amplified polymorphic DNA-PCR analysis, which suggested that genetic changes taking place during the mutagenesis are maintained (Zhang *et al.* 2007).

In order to improve fungal conidiation and phytotoxin production, Zhang *et al.* (2007) used protoplast fusion between the HM1, a mutant derived from HGE with UV radiation treatment, and *Curvularia lunata* to generate new fungal strains with improved biocontrol efficiency. Seven recombined strains showed improved conidia production and four increased production of the phytotoxin ophiobolin A when compared to the HM1 strain.

PHYTOTOXINS

It can sometimes be a challenge to use fungal mycelia or spores for weed control because of environmental constraints under field conditions and/or the need for a broader spectrum of weed control. Therefore, the use of secondary metabolites or phytotoxins produced by bioherbicide fungi has also attracted researchers' attention.

A crude toxin of *E. monoceras* was extracted from the fermentation broth with ethyl acetate (Zheng *et al.* 2008). At 1 g /L, the crude toxin did not affect the germination of barnyard grass significantly, but rather promoted radicle and shoot growth. At 5 g /L, however, the germination of barnyard grass was reduced by 75%, and radicle and shoot growth suppressed by 80%. The greatest efficacy was observed on plants before the two-leaf stage. The toxin may be translocated throughout a barnyard grass plant, but the greatest impact was observed on the root growth. Although the specific mode(s) of action has yet to be elucidated, the highly toxic compound was identified as dibutyl phthalate which mediated the control of barnyard grass.

Duan *et al.* (2007) isolated biologically active metabolites of HGE from mycelia and fermentation broth of the fungus with also ethyl acetate, and found that the crude toxin not only significantly inhibited the growth of barnyard grass but also suppressed the fungus *Rhizoctonia Solani* Kühn, the causal agent of sheath blight disease on rice. Four bioactive sesterterpenoids were purified using chromatography, and the compounds were identified as ophiobolin A, 3-anhydro-ophiobolin B, 3-anhydro-6-epi-ophiobolin A, and 3-anhydro-6-epi-ophiobolin B, respectively, using NMR and MS. Bioassay tests showed that all four metabolites inhibited the radicle of barnyard grass but showed no significant effect on rice or other crop species at concentrations efficacious against the weed. It was later found that the ophiobolin A had the highest efficacy for control of barnyard grass. This compound was also highly inhibitive to the mycelial growth of *R. solani*. In field experiments, applications of crude toxins effectively reduced rice sheath blight, while exerting no adverse effect on the growth and yield attributes of rice plants. HPLC tests indicated no detectable ophiobolin A remained in rice grains for all treatments applied. These results indicate that the metabolites produced by HGE may also be used to protect against sheath blight, an important disease on rice (Duan *et al.* 2006, 2007) in addition to controlling barnyard grass. A micro-emulsion formulation, consisting of a co-solvent, emulsifier, dispersant, and synergistic stabilizer, was developed for delivery of this toxin in rice fields. In field experiments, the treatment of 10% the toxin in micro-emulsion at 300-1,200 g/ha reduced barnyard grass, monochoria grass [*Monochoria vaginalis* (Burm.f.) Presl. Ex Kunth.], false loosestrife (*Ludwigia prostrata* Roxb.), Indian rotala [*Rotala indica* (Willd.) Koehne.], and sedge by 60-80%, with no visible injury to rice plants. When the same formulation was applied at 1,000 g/ha, rice sheath blight was reduced by nearly 80%. This is a rare example where a microbial-base toxin can be used to control primary weed and disease targets in rice fields, and this versatility may boast the benefit of this biocontrol agent.

STRATEGIES FOR DEVELOPING BIOHERBICIDES ON RICE CROPS

There are two important criteria for determining the usefulness of bioherbicide candidates, effectiveness of weed control (efficacy) and host specificity (weed spectrum and crop security). Efficacy has been a challenging aspect for biocontrol of barnyard grass; many research projects did not progress much beyond screening/evaluation stages because some of the critical technologies required for consistent biocontrol performances under variable field conditions are still lacking. Commercial development is slow coming when data are lacking for successful weed control under field conditions. Although some bioherbicide were sold commercially in China, they were generally considered as a supplement to chemical herbicides with a very small market share. There are several constraints to the development of bioherbicides for control of barnyard grass.

Narrow-spectrum of bioherbicides

Highly selective bioherbicide agents are of advantage, as well as disadvantage. For the most part, a single target ensures the crop safety but may be less desirable under conditions where multiple weed species need to be controlled. Therefore, it is difficult to achieve the objective of weed control in a paddy rice field with the bioherbicide only. Application of several host-specific fungal pathogens in a bioherbicide mixture as a multi-component bioherbicide system may address the need for a broader spectrum of weed control (Chandramohan and Charudattan 2003). But it is also a common practice to tank mix different herbicides or mixing herbicides with other pesticides for more effective weed control and more efficient field applications (Christopher *et al.* 2008). There are more than 1,000 recommended herbicide tank mixtures in China, and the list is still growing (Liang *et al.* 2005). Actually these ideas have already been used in several bioherbicide applications. Morin *et al.* (1993) demonstrated synergy between *Puccinia xanthii* Schw. and *Colletotrichum orbiculare* (Berk and Mont.) v. Arx for control of Noogoora burr (*Xanthium occidentale* Bertol.). Chandramohan and Charudattan (2003) showed potential control of pigweed (*Amaranthus retroflexus* L.), sicklepod [(*Dichrostachys cinerea* (L.) Wight & Arn)], and showy crotalaria (*Crotalaria assamica* Benth) simultaneously with a mixture of different fungal pathogens without alteration in host specificity of each fungus. The weeds northern jointvetch [*Aeschynomene virginica* (L.) Britton, Sterns & Poggenb] and winged waterprimrose (*Ludwigia decurrens* Walt.) were controlled by a mixture of *Colletotrichum gloeosporioides* f.sp. *aeschynomene* and *C. gloeosporioides* f. sp. *jussiaeae* (Boyette *et al.* 1979) in rice fields. Furthermore, adding the pathogen *C. malvarum* (A. Braun & Casp.) Southw. to this mixture could effectively control the weed prickly sida (*Sida spinosa* L.) in one spray pass (Smith 1986). These weeds are also problems in paddy rice fields in China. Although it is possible to increase the range of weed control by combining two or more fungal agents, there may be practical challenges for this strategy due to potential costs associated with registration of multiple agents. It may be more cost effective to tank mix a bioherbicide with a broad-spectrum synthetic herbicide to target a hard-to-control weed problem in a cropping system.

Mixtures of a pathogenic fungus with reduced rates of chemical herbicides may enhance the efficacy while expand the spectrum of weed control simultaneously. This strategy may also significantly decrease the amount of herbicide use. For these reasons, the strategy has been suggested as one of the promising ways to integrate weed biocontrol in cropping systems (Boyette 2006; Graham *et al.* 2006; Boyette *et al.* 2008). Brooker *et al.* (1996) combined the natural tripeptide herbicide bialaphos with a strain of *C. gloeosporioides* f.sp. *aeschynomene* genetically engineered to resist this herbicide for improved control of both northern and Indian jointvetch. Smith and Hallett (2006) demonstrated that only half the recommended label rate of glyphosate was needed for control of common waterhemp [*Acnida tamariscina* (Nutt.) A. Wood] when combined with the fungus *Microsphaeropsis amaranthi* (Ellis & Barthol.) Heiny & Mintz and applied within 1-3 days of herbicide treatment. In a different study, application of either *C. graminicola* or *Gloeocercospora sorghi* Bain & Edgerton ex Deighton with or 1-3 days prior to a sub-lethal concentration of glyphosate caused antagonism for control of shattercane [*Sorghum bicolor* (L.) Moench] by the fungus, while spraying glyphosate prior to the fungal agents resulted in an increased level of weed control (James *et al.* 2008). Larger barnyard grass plants, 22 and 30 days after seeding, were killed by a combination of *Cochliobolus lunatus* and sublethal rates of atrazine, compared to moderate leaf damage to plants treated with either the fungus or herbicide alone (Smith 1991). However, the bioherbicide Collego™ (*Colletotrichum gloeosporioides* f. sp. *aeschynomene*) may only be tank mixed with a limited number of synthetic herbicides because many of the chemicals used in rice fields inhibited the germination and growth of the fungus (Klerk *et al.* 1985).

Some soil-borne pathogens with a broad range of hosts may be of value for controlling a variety of weeds at pre-emergent or post-emergent stages (Boyetchko and Peng 2004; Medd and Campbell 2005). It may also encounter fewer environmental constraints than foliar-applied bioherbicide agents because soil conditions are generally less fluctuating. However, the poor selectivity of many soil-borne pathogens may limit their application due to crop safety concerns.

Environmental constraints

Fastidious environmental requirements may also be a major limitation to bioherbicide development. Often a long dew period and/or high relative humidity are two key factors to the performance of many bioherbicides agents, especially to those applied as a foliar treatment. In paddy rice fields, only the dew period is critical while in directly seeded rice fields, the humidity level can sometimes be a constraint for germination and infection by the bioherbicide agent. Possibly, formulation holds the key to biocontrol of barnyard grass with HEG or *E. monoceras* in paddy rice fields where often a slight extension of the dew period makes substantial impact on successful infection (Zhang *et al.* 2007). Collego^{TM} was formulated in a water-soluble polysaccharide alginate gel powder which may have contributed to its effectiveness in controlling north joint vetch in rice fields in southern USA (Mortensen 1998). The formulation may make the micro environment on the leaf surface less hostile to the fungal biocontrol agent.

Technological improvements – microbial mass production and formulation

Lagging formulation technology is another major limiting factor for development and application of bioherbicides. Although a micro-emulsion of HGE crude toxins looked promising to weed control in field trials, its application is still limited by cost and other factors and, as a result, commercial development has not been initiated. Formulation technologies remain a priority for improvement of bioherbicide agents. Advancements have been made in the formulation and application of bioherbicide agents, including the use of alginate gel technology, controlled release formulations of active ingredients based on microencapsulation (Sopeña 2009), invert emulsions (Auld 1993; Boyette *et al.* 1993; Rosskopf *et al.* 2005) and various additives (Weaver *et al.* 2009) to enhance germination, virulence, and efficacy. However, further improvements are required to make these technologies practical for field applications in terms of low cost and ease of use.

Microbial herbicides often consist of living organisms as active ingredients, so they are generally unstable under UV light, extreme temperatures and/or desiccation conditions. Without protection, they may quickly lose their biological activity after field application. This is also an inherent challenge to nearly all bioherbicides, which must be overcome before a bioherbicide can be widely acceptable for practical uses. Some of the recent advances in adjuvant technologies, spraying and delivery systems have been explored to address these limitations. An oil-in-water emulsion of unrefined corn oil and Silwet L-77 increased the biological weed control by *Colletotrichum truncatum* (Schwein.) Andrus and W.D. Moore for control of hemp sesbania [*Sesbania exaltata* (Raf.) Cory]. The surfactant – corn oil emulsion stimulated germination and appressorial formation and alleviated the need for long dew by the fungus (Boyette *et al.* 2007). In field experiments conducted over three years, a single application of *C. truncatum* in 50% (v/v) unrefined corn oil emulsion containing 0.2% (v/v) Silwet L-77 surfactant controlled hemp sesbania in soybeans by an average of 95%. At 1: 1 (v/v) mixing ratio, aqueous suspension of *C. gloeosporioides* and corn oil mixture containing the same surfactant reduced the dew period requirements for maximum weed infection and mortality from 16 to 8 h, and delayed the need for free moisture for greater than 48 h after application (Boyette 2006). This formulation also enabled the pathogen to infect and kill weeds in more advanced growth stages (Boyette 2006). These results indicate that formulating fungal bioherbicide agents improves weed control efficacy under field conditions, and equipping the agents HEG or *E. monoceras* with a proper formulation technology may substantially increase the efficacy and consistency of barnyard grass control in rice fields.

Lagging research in fermentation technologies and high costs has made mass production of barnyard grass bioherbicide agents difficult, delaying the development of these agents into commercial products. Liquid fermentation and solid-state fermentation are the main cell mass production methods, and the liquid process is often the preferred method for production of microbial herbicides because the technology more sophisticated and process can be controlled more easily (Avid 1995). Some fungi do grow slowly and sporulate poorly in these fermentation systems and sometimes the virulence may decrease after mass propagation. All these factors affect mass production and commercialization potential of a bioherbicide candidate. Optimization of spore yields ("sporulation") is often a critical aspect in determining the success or failure of a bioherbicide mass production and commercial prospect (Boyette *et al.* 1991). For example, commercial production of the bioherbicide LUBAO in China was hindered by degradation of infectious propagules during the process of mass propagation. Optimization of nutrients in the medium and fermentation environment is critical to the improvement of biomass yield and potency (Babu *et al.* 2004) and significant progress may be made with enhanced research in fermentation technologies targeting specific agents. In China, both liquid and solid fermentation were used alternately to differentially stimulate mycelial growth and conidiation of *Alternaria alternata*, a bioherbicide agent for Crofton weed [*Ageratina adenophora* (Spreng.) R.M.King & H.Rob]. A method of pressure pulse solid fermentation technology was first launched at the Chinese Academy of Sciences to stimulate sporulation of certain fungi. Some of these fermentation technologies are maturing and near prototype industrial models are being validated. These new technologies may be tested for mass production of the bioherbicide agents HEG and *E. monoceras*.

FUTURE PERSPECTIVES

Although the research on biocontrol of barnyard grass is still in an early phase, promising bioherbicide agents have been identified, and initial results from laboratory and field trials are encouraging. Prospects for the development and utilization of this technology are good due to renewed support to the research from several major granting agencies and increased public awareness of environmental issues. Recently, the government of China substantially increased the funding for bioherbicide research through a national 863 Program. To be successful in developing biocontrol for barnyard grass, continued efforts are needed to improve the virulence and efficacy of HGE and *E. monoceras* under field conditions. Fermentation and formulation technologies will be the key to efficient mass production and practical shelf live and delivery, and major progress in these areas will help facilitate commercial development of these agents. Industry participation may be solicited at this stage to fast track the development because of existing technological capabilities and the need for foresights in regulatory processes business development further down the road.

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