

# IX<sup>th</sup> INTERNATIONAL BIOHERBICIDE GROUP WORKSHOP



**ORLANDO, FLORIDA, USA**  
**February 8, 2009**

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Research Initiative**



**Also sponsored by:**



## **PREFACE**

### **Introduction**

Welcome to the IX<sup>th</sup> Workshop of the International Bioherbicide Group (IBG) at the Hilton Walt Disney World hotel in Orlando Florida, 8 February 2009. This workshop was organized as a satellite workshop in association with the Weed Science Society of America annual meeting in order to foster greater communication and collaboration between the “traditional” weed science community and IBG researchers. The IBG provides an informal forum for communication and collaboration among scientists with a common interest in the utilization of microorganisms in the management of weeds. All interested individuals are invited to participate and to subscribe to the IBG news. To subscribe to the IBG news and be placed on the IBG email list, please go to the IBG newsletter website: <http://ibg.ba.cnr.it/>. We hope that the workshop will be a valuable experience for all delegates.

### **Delegates**

There is a list of workshop participants who pre-registered and their email addresses at the end of this document. At last count, this included 46 people from 12 countries.

### **Organizing Committee**

Joe Neal, Department of Horticultural Science, North Carolina State University, Raleigh, NC 27695 USA

John Lydon, U.S. Department of Agriculture, Agricultural Research Service, Sustainable Agricultural Systems Laboratory, Bldg. 001, Rm. 227, Beltsville, MD 20705 USA

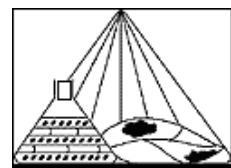
William L. Bruckart, III, USDA, ARS, Foreign Disease-Weed Sci. Res. Unit, 1301 Ditto Ave. Ft. Detrick, MD 21702 USA

### **Proceedings Editors**

J. Pablo Morales-Payan, Department of Horticulture, University of Puerto Rico-Mayaguez Campus, and Joe Neal, Department of Horticultural Science, North Carolina State University, Raleigh.

### **This document**

This booklet is the Proceedings of the IX<sup>th</sup> IBG Workshop. It contains the workshop program and the summaries of the oral and poster presentations in the workshop.



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IXth International Bioherbicide Group Workshop  
 Sunday, 8 February 2009  
 Program

8:00	Registration	
8:45	Welcome and Introductions	G. W. Bourdot and J. C. Neal
9:00	Efficacy of biological control of grassy weeds using <i>Curvularia eragrostidis</i> in the field trials	Y. Zhu, T. Gao, J. Wang, Y. Lu, S. Qiang*; Nanjing Agricultural University, Nanjing, China
9:15	Evaluation of bioherbicidal control of tropical signalgrass, <i>Urochloa subquadriflora</i>	Y. M. Shabana*, C. Stiles, R. Charudattan A. Abou Tabl, J. White, University of Florida, Gainesville, FL, Valdosta State University, Valdosta, GA USA
9:30	Screening fungal pathogens of <i>Microstegium vimineum</i> as potential biocontrol agents.	L. C. Walker*, J. C. Neal, L. P. Tredway; North Carolina State University, Raleigh, NC USA
9:45	The Model of Conidia Production in <i>Helminthosporium</i> spp., Biological Agents for Grassy Weeds Control.	K. Yamaguchi*; Minami Kyushu University, Takanahe-cho, Japan
10 - 10:30	Discussion and Break	
10:30	A search for a root-pathogen of <i>Cirsium arvense</i> in New Zealand	G. W. Bourdot* B. Skipp, G. Hurrell, D. Saville; AgResearch Limited, Christchurch, New Zealand, AgResearch Limited, Palmerston North, New Zealand, Saville Statistical Consulting Limited, Christchurch, New Zealand
10:45	Root Colonization and Environmental Fate of the Bioherbicide <i>Pseudomonas fluorescens</i> BRG100	S. M. Boyetchko*, C. Hanson, R. K. Hynes, D. Korber; Agriculture and Agri-Food Canada, Saskatoon, SK, Canada, University of Saskatchewan, Saskatoon, SK, Canada
11:00	Herbicide- deleterious rhizobacterial interactions in Velvetleaf weed control.	R. Zdor*; Andrews University, Berrien Springs, MI USA
11:15	Using Gene Fusions to Study Cyanogenesis in a Weed Deleterious Rhizobacterium	M. M. Biswas*, R. Zdor, C. Miller; Andrews University, Berrien Springs, MI USA
11:30	Discussion	
11:45 -	LUNCH BREAK	
1:15	Evaluation of <i>Alternaria alternata</i> as a potential biocontrol agent for field bindweed ( <i>Convolvulus arvensis</i> )	Ehsan Zeydali, Alireza Koocheki, Nader Azadbazhtl, Mohammad H. Rashed and Reza Ghorbani*, Lorestan Res. Inst. of Agric. & Natural Resources, Khoramabad, and Ferdowsi Univ. of Mashhad, Mashhad, Iran
1:30	Fungal toxins and other natural metabolites for management of parasitic weeds	M. Vurro*, A. Boari; National Research Council, Bari, Italy
1:45	Pathogenic Mechanisms of Vulculic Acid Produced by <i>Nimbya alternantherae</i>	M. Xiang*, L. Fan, Z. Jiang, Y. Zeng; Zhongkai University of Agriculture and Engineering, Guangzhou, China, South China Agricultural University, Guangzhou, China
2:00	Surfactants affect the efficacy of <i>Alternaria cassiae</i> controlling sicklepod seedlings	R. A. Pitelli*, C. F. Franco, F. M. Claudia; University of State of Sao Paulo, Jaboticabal, Brazil

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2:15	Discussion and break	
2:45	Potential of <i>Cassida rubiginosa</i> as a biological control agent for <i>Cirsium arvense</i>	Ghorbanali Assadi, Reza Ghorbani, Mohammad H. Rashed and Hossein Sadeghi; Ferdowsi Univ. of Mashhad, Mashhad, Iran
3:00	Native Phytopathogens as Biocontrol Agents: Problems and Potential in the Management of Invasive Exotic Species	K. Jayachandran*, K. G. Shetty; Florida International University, Miami, FL USA
3:15	Media Studies on <i>Myrothecium roridum</i> Tode (IMI 394934); a Potential Biocontrol Agent for Water Hyacinth.	Okunowa Wahab Oluwanisola, G.O. Gbenle, A. A. Osuntoki, and A. A. Adekunle, University of Lagos, Nigeria
3:30	Bioherbicide development against water hyacinth: the story so far and the hopes for the future	R. W. Barreto*, D. J. Soares, E. M. Inokuti; Universidade Federal de Viçosa, Viçosa, MG, Brazil
3:45	Status of <i>Phoma macrostoma</i> , a bioherbicide for broadleaved weed control in turfgrass.	K. L. Bailey*, S. Falk, S. Lombardo; Agriculture & Agri-Food Canada, Saskatoon, SK, Canada, The Scotts Company, Marysville, OH USA
4:00	Discussion	
4:30	Business Meeting	
5:30 - 7:00	IBG Reception at Wolfgang Puck Restaurant	Sponsors: Novozymes Biologicals, Inc, The Scotts Company, and SePRO Corporation

### Poster Presentations

	Title	Authors
	Effects of <i>Phomopsis amaranthicola</i> on the Above- and Below-ground Interference of Pigweeds with Bell Pepper.	J. Morales-Payan*, R. Charudattan, W. M. Stall; University of Puerto Rico-Mayaguez Campus, Mayaguez, PR, University of Florida, Gainesville, FL USA
	Implementing successful biological control of Canada thistle with the host-specific rust fungus: <i>Puccinia punctiformis</i>	By BERNER, D.K., BACKMAN, P.A., SHEA, K., and CONAWAY, S.A. Dana K. Berner, Plant Pathologist Leader Biological Control of Weeds CRIS project, USDA, ARS, Foreign Disease-Weed Science Research Unit 1301 Ditto Ave., Ft. Detrick, MD 21702-5023 USA

**Efficacy of biological control of grassy weeds using *Curvularia eragrostidis* in the field trial**

Y. Zhu, T. Gao, J. Wang, Y. Lu, S. Qiang\*; Nanjing Agricultural University, Nanjing, China

*Curvularia eragrostidis* is being developed as part of a biologically based weed management tactic for control of grassy weeds in China. A large number of field trials were conducted between 2004 and 2008 on two NJAU research field sites near Nanjing to determine efficacy of the fungus, in varying applications, for control of large crabgrass (*Digitaria sanguinalis*) under different field conditions. Treatments included different rates of fungal conidia, reduced rates of herbicides, and mixtures of the fungus with herbicides at reduced rates. The fungus applied at  $5 \times 10^5$  conidia/ml and 700 L/ha generally achieved significant weed suppression (> 50% weed mortality) under a range of field conditions. Weather conditions as well as growth stage of the weed influenced the efficacy of the fungus; under favorable weather conditions such as frequent rain during the growing season in 2006, the fungus controlled large crabgrass at the 2.0-3.0 leaf stage by up to 90% when compared to untreated controls. Biocontrol efficacy and consistency was improved by adding adjuvants or humectants in spray formulations. When *C. eragrostidis* ( $5 \times 10^5$  conidia/ml) was tank-mixed with nicosulfuron at reduced rates (0.2X or 0.1X recommended rate), the fungus interacted synergistically with the herbicide and controlled large crabgrass consistently by 75% in soybean and corn fields even under dew-free conditions. The mixture was significantly more effective than either single-agent treatment. These results indicate that *C. eragrostidis* is a promising mycoherbicide agent for control of large crabgrass. Herbicides at substantially reduced rates may be used with the fungus as a synergizer to enhance the efficacy and consistency of weed control while decrease the chemical load in the environment.

**Evaluation of bioherbicidal control of tropical signalgrass (*Urochloa subquadrifera*)**

Yasser M. Shabana\*, Univ. of Florida, Gainesville; Carol Stiles, Valdosta State Univ., Valdosta, GA; Raghavan Charudattan, Ayman H. Abou Tabl and Jodi White, Univ. of Florida, Gainesville. E-mail: yassershhabana2@yahoo.com

Key words: tropical signalgrass, biological control, bioherbicides, pelargonic acid, ammonium sulfate.

Tropical signalgrass (TSG), aka alexandergrass causes serious problems for sod production and turf maintenance in Florida. Other grasses such as crabgrass (*Digitaria sanguinalis*; CG), smut grass (*Sporobolus indicus*, SG), thin paspalum (*Paspalum setaceum*, TP), and torpedograss (*Panicum repens*, TG) can also be problematic. In an attempt to develop a bioherbicide for these grasses, the foliar fungal pathogen *Drechslera gigantea* (Dg), which has been reported as a promising biocontrol agent for several grass weeds, was evaluated. Formulations composed of Dg mycelium and/or Dg culture filtrate, both produced in a liquid broth, and Sunspray 6E oil were tested with or without ammonium sulfate (a foliar desiccant and fungal nutrient) or pelargonic acid (N-nonanoic acid; a natural product and a registered biorational herbicide) in greenhouse and field trials. Two solid formulations composed of Dg mycelium entrapped in natural hydrophilic polymers or wheat gluten and kaolin were also tested in greenhouse trials. A 30% Sunspray 6E oil formulation containing Dg mycelium (10 g), Dg culture filtrate (70 ml), and 4.5 g ammonium sulfate caused 88% to 100% damage on TSG, CG, SG, and TG in greenhouse trials. The damage from this formulation was the result of disease as well as phytotoxicity from the culture filtrate, oil, and ammonium sulfate. Compared to the emulsion formulations, the solid formulations did not provide satisfactory results. An emulsion formulation containing 30% Sunspray 6E oil and 70% culture filtrate free of Dg mycelium to which was added 2% (v/v) pelargonic acid caused 100% damage to SG 2 weeks after application. Grasses treated with Dg formulations containing ammonium sulfate or pelargonic acid and without exposure to a dew period showed higher levels of damage compared to those treated with the same formulations and given a 24-h dew period. Formulations containing Dg mycelium, Dg culture filtrate, and ammonium sulfate or pelargonic acid are effective and promising for control of weedy grasses. Further evaluation of these formulations under field conditions is justified.



**Screening fungal pathogens of *Microstegium vimineum* as potential biocontrol agents**

Walker, L.C.1\*; Neal, J.C.1; Tredway, L.P.2  
1Department of Horticultural Science, North Carolina State University, Campus Box 7609, Raleigh, NC, 27695  
2Department of Plant Pathology, North Carolina State University, Campus Box 7616, Raleigh, NC, 27695  
lcwalker@ncsu.edu

**Abstract**

Japanese stiltgrass (*Microstegium vimineum*) is an invasive summer annual grass in the eastern United States. Although there are herbicides available that control Japanese stiltgrass, many of the invaded sites are environmentally sensitive areas where control options are limited. In 2003 and 2004, a pathogen was observed on Japanese stiltgrass seedlings in North Carolina that caused zonate leaf spots, leaf tip necrosis, and eventual plant death. In 2005 and 2006, samples collected from these sites were identified as *Drechslera gigantea*. In 2007 and 2008, samples from diseased seedlings from the same sites demonstrating similar symptoms were collected and the fungi isolated in culture. Five isolates were collected in 2007 and seven were collected in 2008; however, most of these were determined to be non-pathogenic. Two greenhouse trials were conducted to screen the isolates for pathogenicity on seedling Japanese stiltgrass. Three isolates were determined to be pathogenic and caused similar symptoms to those observed in the field. The isolates were identified as *Curvularia* (two isolates) and *Drechslera*. No *D. gigantea* was recovered in 2007 or 2008. Efforts are continuing to identify these isolates to species and evaluate their potential as biological control agents for Japanese stiltgrass.

**Introduction**

Japanese stiltgrass (*Microstegium vimineum*) is a weedy C4 grass commonly found throughout the eastern United States in shady, moist areas such as woodlands, floodplains, drainage ditches, riverbanks, landscapes and turf. The invasive nature of this weed often displaces native plant populations. Management of this weed is difficult because infestations are often too extensive for hand removal to be feasible and few herbicides are labeled for use in the typical invaded ecosystems. In 2003 and 2004, a pathogen was observed on Japanese stiltgrass seedlings in North Carolina that caused zonate leaf spots, necrosis of the terminal leaves, and eventual plant death. In 2005 and 2006, samples collected from these sites were identified as *Drechslera gigantea*. *D. gigantea* is a pathogen of the Poaceae family, demonstrating distinct zonate eye-spots on vegetation (Hyde et al. 1992). This pathogen has also been identified and screened for pathogenicity on various weedy grasses with measured success in Canada and Florida (Green et al. and Chandramohan et al.). The overall objective of this project was to isolate endemic pathogens of Japanese stiltgrass and evaluate these pathogens for their potential as biological control agents for this invasive grass weed. Specifically we hoped to recover isolates of *D. gigantea* and other endemic pathogens of Japanese stiltgrass and test these isolates for efficacy in the control of Japanese stiltgrass.

**Materials and methods** In July 2007, leaf samples displaying leaf spots were collected from Harris Lake Country Park and along Lead Mine Creek in Wake County, NC, where seedlings infected with *D. gigantea* had been observed in previous years. Five isolates were obtained, but all were determined to be non-pathogenic fungi. In May 2008, seedlings of Japanese stiltgrass displaying zonate eyespots and necrosis of the terminal leaf were collected from Harris Lake Country Park and Schenck Memorial Forest in Wake County, NC. Seven isolates were collected including a *Drechslera* isolate and two *Curvularia* isolates that have yet to be identified at the species level. The remaining isolates were determined to be non-pathogenic or saprophytic species. The *Drechslera* cultures were grown and maintained on

carrot agar at ambient temperature and continuous light. The *Curvularia* cultures were grown and maintained on potato dextrose agar (PDA) at ambient temperature and light. In September 2008, spore suspensions were prepared for the *Curvularia* and the *Drechslera* isolates. The spore suspensions for *Drechslera* measured  $5.0 \times 10^3$  conidia/mm<sup>2</sup> and *Curvularia* measured  $1.8 \times 10^6$  conidia/mm<sup>2</sup>. Japanese stiltgrass seedlings were grown in 4-inch plastic pots containing Fafard 2P potting media, in greenhouse conditions. Three single-pot replications were included for each isolate and a nontreated control. Spore suspensions and the nontreated control (distilled water) were applied to Japanese stiltgrass seedlings at the 4-leaf stage using an airbrush sprayer. Treated pots were placed in high humidity and total darkness for 48 hours. The pots were then moved into a greenhouse. Visual ratings of percent leaf area displaying symptoms using the Horsfall-Barratt scale (1-12) were recorded 2, 4, 6, and 8 days after treatment (DAT). The organisms were then re-isolated in culture from the leaves displaying symptoms, and identified. In December 2008, spore suspensions for the *Drechslera* isolate and the more virulent *Curvularia* isolate #1 were prepared with distilled water and applied similarly as in the first experiment. Additional suspensions were prepared with Triton X-100 (octylphenol ethylene oxide condensate) at 0.25% v/v for both *Drechslera* and *Curvularia* as well as a nontreated control (distilled water + 0.25% Triton X-100). Spore suspensions of *Drechslera* treatments measured  $3.2 \times 10^4$  conidia/mm<sup>2</sup>, and  $2.2 \times 10^5$  conidia/mm<sup>2</sup> for the *Curvularia* treatments. Visual ratings of percent leaf area displaying symptoms were recorded 2, 4, 6, and 10 DAT.

**Results and discussion** In the first trial, damage from the *Drechslera* isolate was a maximum of 3 on the Horsfall-Barratt scale (3-6%), for *Curvularia* isolate #1 a maximum of 5 (12-25%), and for *Curvularia* isolate #2 a maximum of 2 (0-3%) (Fig. 1). Symptoms for *Drechslera* included zonate leaf spots that would coalesce and girdle the leaf. For the *Curvularia* isolate #1, symptoms included zonate leaf spots and necrosis of the youngest leaf. Symptoms for the *Curvularia* isolate #2 were minimal, consisting of only a few leaf spots. In the second trial, damage from the *Drechslera* isolate applied in water measured a maximum of 5 on the Horsfall-Barratt scale, and *Curvularia* a maximum of 2 (0-3%) (Fig. 2). Symptoms for the *Drechslera* isolate consisted of many small, dark leaf spots and some larger, zonate leaf spots. As observed in the first experiment, symptoms from *Curvularia* inoculation were minimal, consisting of only a few leaf spots. Triton X-100 applied at 0.25% in water caused significant foliar necrosis, with severity ratings of 4 to 5, similar to that caused by the *Drechslera* + water inoculation. Symptoms of damage for this treatment included water soaking and later necrosis of the affected tissues. Triton X-100 combined with *Curvularia* or *Drechslera* spore suspensions resulted in damage ratings of about 9 (87-94%) for both isolates. Symptoms for both treatments included water-soaking of leaf tissue coinciding with many leaf spots and necrosis of the terminal leaf. At 10 DAT, symptoms included total plant death of most seedlings. After 8 DAT for both trials, surviving seedlings had new growth present that was apparently unaffected by treatments. These tests demonstrated that field observations of leaf spot and plant necrosis can be replicated with endemic isolates of *Drechslera* and *Curvularia*. Under experimental conditions Japanese stiltgrass control is enhanced by the addition of an adjuvant. Further testing is needed as results varied between trials and additional surfactants and additives should be tested. In addition, spores suspended in solutions containing these additives should be tested for germination. Additional organisms will also be surveyed for potential use as biological control agents of Japanese stiltgrass.

## References

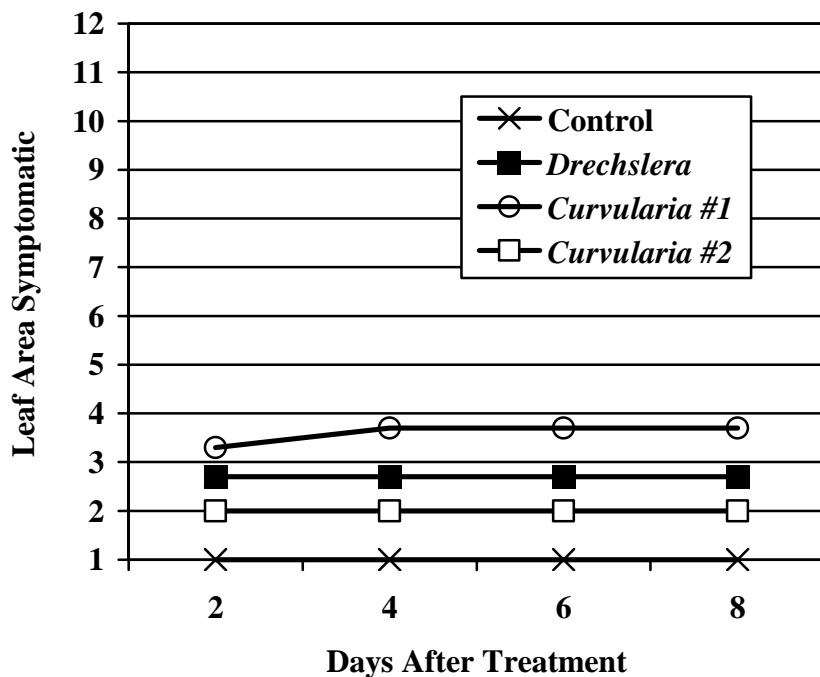
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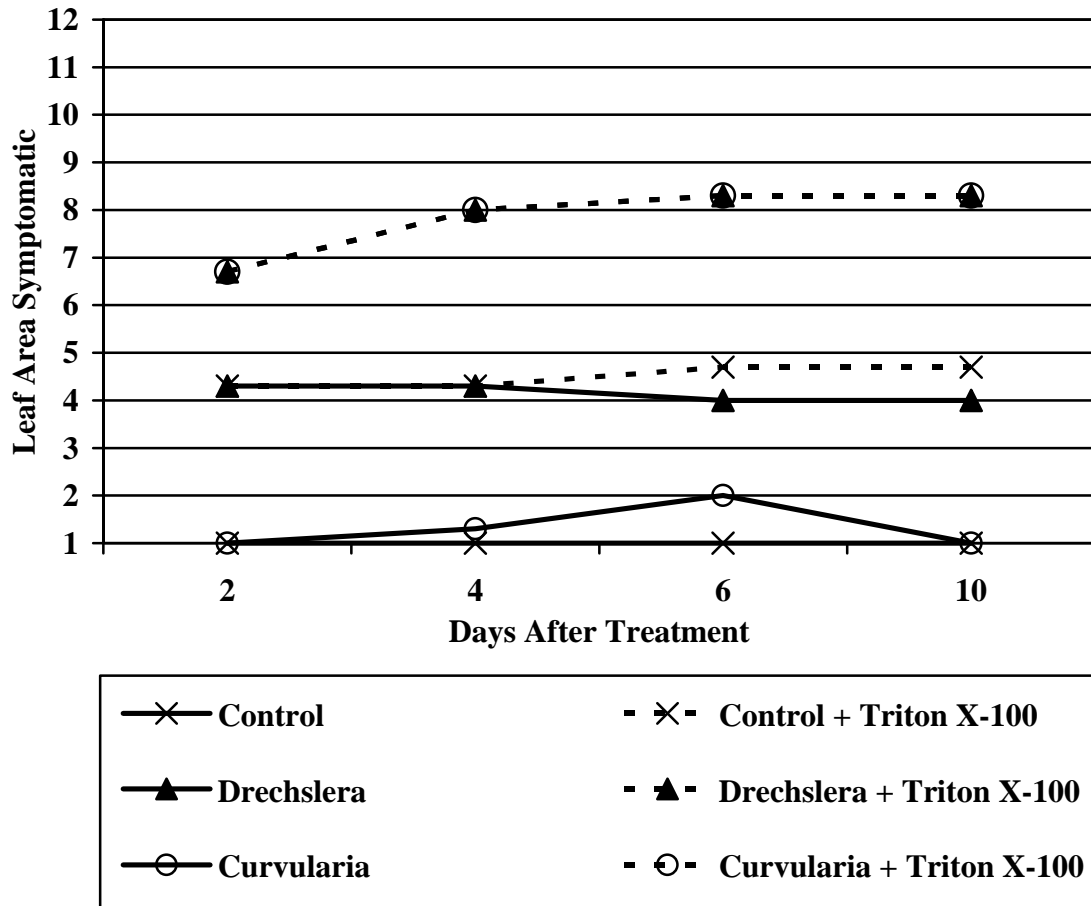
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**Fig. 1.** Mean leaf area displaying symptoms of Japanese stiltgrass infected with *Drechslera* and *Curvularia* isolates, 1<sup>st</sup> trial. Ratings on Horsfall-Barratt scale, 1-12.



**Fig. 2.** Mean leaf area displaying symptoms of Japanese stiltgrass infected with *Drechslera* and *Curvularia*, 2<sup>nd</sup> trial. Ratings on Horsfall-Barratt scale, 1-12.



## **The model of conidia production in *Helminthosporium* species, biological agents for grassy weeds control**

K. Yamaguchi

Lab for Sustainable Horticulture, Minami Kyushu University, Takanahe-cho, Miyazaki 884-0003, Japan \*keny@nankyudai.ac.jp

### **Abstract**

Several fungal isolates of *Helminthosporium* species have been reported as promising biocontrol agents against grassy weeds. *Exserohilum rostratum* which shows herbicidal activity against red sprangletop (*Leptochloa chinensis*) can produce conidia only in the air, not in any liquid medium. The methods of a two-phase system, using sponge matrix and via mycelial pellets, were examined for conidia production of this fungus. In the first phase, quantitatively sufficient mycelia were obtained in submerged liquid culture. In the second phase, abundant conidia were formed when the mycelia was exposed to the air. The two-phase system both using sponge matrix and via mycelial pellets consistently produced over  $10^8$  conidia L<sup>-1</sup>. In addition, the conidia were more uniform in size, and showed higher percentage of germination, than those produced by plate culture. These results suggested the two-phase system described in this study could be a prototype of mass-production of a bioherbicide based on *Helminthosporium* species.

### **Introduction**

There have been several publications in which *Helminthosporium* species show potential efficacy as a bioherbicide against grassy weeds, for example barnyardgrass and red sprangletop (Yamaguchi 2006). Mass production and formulation seem to be key technologies to develop bioherbicides based on *Helminthosporium*. However, the fungus *Helminthosporium* including *Bipolaris*, *Drechslera*, and *Exserohilum* does not produce any conidia in liquid medium. In this study two methods of a two-phase system for conidia production, using sponge matrix and via mycelial pellets, were examined by use of *E. rostratum* which shows herbicidal activity against red sprangletop.

### **Materials and methods**

**Fungal isolate.** The *Exserohilum rostratum* (*Setospharia rostrata* in teleomorph) isolated from diseased red sprangletop in Kyushu, Japan, was used as a representative of *Helminthosporium*. This fungus was reported as a promising biocontrol agent against red sprangletop in both Japan (Yamaguchi et al. 2005) and Vietnam (Chin et al. 2003).

**Two-phase system on conidia production.** Procedures of a two-phase system for synchronous conidia production are schematically presented in Fig.1. In the first phase, quantitatively sufficient mycelia were cultured in submerged liquid culture. The basal medium contained 25g of glucose, 2g of NaNO<sub>3</sub>, 0.5g of K<sub>2</sub>HPO<sub>4</sub>, 0.5g of KH<sub>2</sub>PO<sub>4</sub>, 0.5g of MgSO<sub>4</sub>, 0.2g of CaCl<sub>2</sub>, 3.4 g of polypeptone, 3.4g of yeast extract, and 20g of rice oil per 1 L of distilled water. In case of using sponge matrix 20g of polyurethane foam (5mm-cubes, Bridgestone Ltd. Tokyo) was inserted into liquid medium as a biomass support particle. In case of via mycelial pellets 0.002% Rose Bengal (Wako, Ltd. Osaka) was added in medium. Each 500 mL-flask containing 150 mL of the medium was inoculated with 0.1 % conidia suspension ( $10^4$  conidia mL<sup>-1</sup>) obtained by plate culture. These flasks were incubated on a rotary shaker at 100 rpm in the dark at 25 C.

In the second phase, the formed mycelia was separated from the culture broth through filtration using a sieve. To cause the conidiation of the fungus, the mycelia were exposed to the air in a beaker at 25 C under moist condition. The conidia were harvested with distilled

water containing 0.05 % Triton X-100 using a magnetic stirrer, then filtrated, and kept in suspension.

## Results and discussion

**Effect of the two-phase system using sponge matrix.** In the first phase, hyphae of *E. rostratum* grew well, and formed thin mycelia on the surface of the polyurethane foam in the liquid medium. The mycelia on polyurethane foam were exposed to the air, and so conidia were being produced from the mycelia in the second phase. The number of conidia produced in 1L of liquid medium was over  $10^8$  conidia.

**Effect of the two-phase system via mycelial pellets.** The conidia of *E. rostratum* applied as seeds were germinated, and then being formed 2-3mm mycelial pellets in the liquid medium. The mycelial pellets reached a maximum yield of over 10 dried g L<sup>-1</sup>. These pellets were exposed to the air and consequently enough conidia were produced on the surface of pellets.

The conidia obtained by the two-phase system both using sponge matrix and via mycelial pellets were more uniform in size, and showed higher percentage of germination, than those produced by plate culture. The characteristics of conidia produced by the two-phase system seem to be more suitable for formulation. The two phase-system described here could be a prototype of mass-production of a bioherbicide based on *Helminthosporium* species.

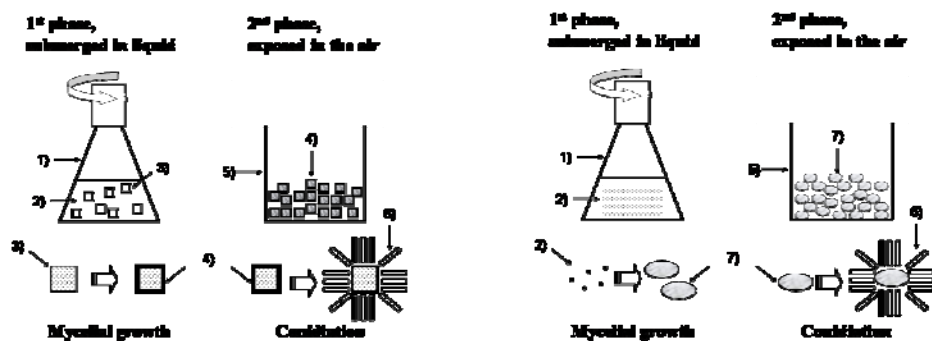


Fig.1

Schematic illustration of a two-phase system. Left, using sponge matrix; right, via mycelial pellets. 1) flask; 2) liquid medium including fungus; 3) polyurethane foam; 4) mycelia on the surface of polyurethane foam; 5) beaker; 6) formed conidia; 7) mycelial pellets.

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### **A search for a root-pathogen of *Cirsium arvense* in New Zealand**

Bourdôt, G.<sup>1\*</sup>; Skipp, R.<sup>2</sup>; Hurrell, G.<sup>1</sup>; Chen, L-Y<sup>2</sup>; Wilson, D<sup>3</sup>; Saville, D.<sup>4</sup>

<sup>1,2,3</sup>AgResearch Limited, <sup>1</sup>PB 4749, Christchurch 8140, New Zealand; <sup>2</sup>PB 11008,

Palmerston North, New Zealand; <sup>3</sup>PB 3123 Hamilton, New Zealand; <sup>4</sup>Saville Statistical Consulting Limited, 147 Bells Rd, West Melton, No. 1 R.D., Christchurch 7671, New Zealand

\*[graeme.bourdote@agresearch.co.nz](mailto:graeme.bourdote@agresearch.co.nz)

#### **Abstract**

The plant pathogenic fungus, *Verticillium dahliae*, is geographically widespread in pasture populations of *Cirsium arvense* in New Zealand, occurring in diseased shoots collected on 42% of randomly sampled farms in a national survey in 2005-06. Its life history suggests it could be the cause of the demise of *C. arvense* populations when they are mown in the rain (a phenomenon reported by farmers). This hypothesis is being tested in field experiments.

#### **Introduction**

*Cirsium arvense* (L.) Scop. (Californian thistle, Canada thistle, creeping thistle) is a troublesome weed in crops and pastures in NZ. In pastures, production losses, the facilitation of “scabby mouth” disease in livestock, and the costs of control measures (mainly herbicides and mowing that typically give only temporary control), justify the ongoing search for an effective control method. To that end the NZ government together with the dairy, sheep and beef farming industries and local community groups have funded a variety of projects aimed at developing “classical” (Gourlay, 2004) and “inundative” bio-control solutions (Bourdôt *et al.*, 2006). To date neither of these approaches has been successful. To effectively control established populations of *C. arvense* in pasture, any control agent must debilitate the weed’s creeping root system since it is the adventitious buds on these roots that drive population growth (Bourdôt *et al.*, 2006). The bio-control approaches to date have attempted to do this by attacking the aerial shoots, an approach that could in theory work since the size of an overwintered root bud population, and hence next season’s aerial shoot population, is directly related to shoot biomass duration in the current growing season (Bourdôt *et al.*, 1998). Here we consider the alternative approach of directly targeting the roots. We (1) describe a field survey to find root-attacking pathogens naturally occurring on *C. arvense* in NZ pastures, and (2) consider the bio-control potential of one fungus found in that survey.

#### **Material and Methods**

The Land Cover Database of NZ, versions 1 and 2 (LCDB1 & LCDB2), were used to locate and map the land in permanent pasture across the entire surface of NZ. A total of 128 sampling points were randomly located within the area of permanent pasture, distributed among the 16 geo-political regions (Nelson and Marlborough combined giving 15 “survey” regions) in proportion to the area of pasture and expected frequency of *C. arvense* in each. Diseased *C. arvense* shoots were sampled at 31 points in spring 2005 and 97 in autumn 2006. Twenty additional samples of diseased *C. arvense* shoots were received from farmers in response to publicity (15 in spring and 5 in autumn), giving a total of 148 samples of disease shoots from throughout NZ. For each sample (8 shoots) the disease symptoms were recorded and appropriate isolations were made onto antibiotic water agar from surface-sterilised symptomatic foliage tissue and from vascular tissues from stems and roots. Some of the isolates were applied to healthy *C. arvense* plants and were re-isolated, completing Koch’s four postulates.

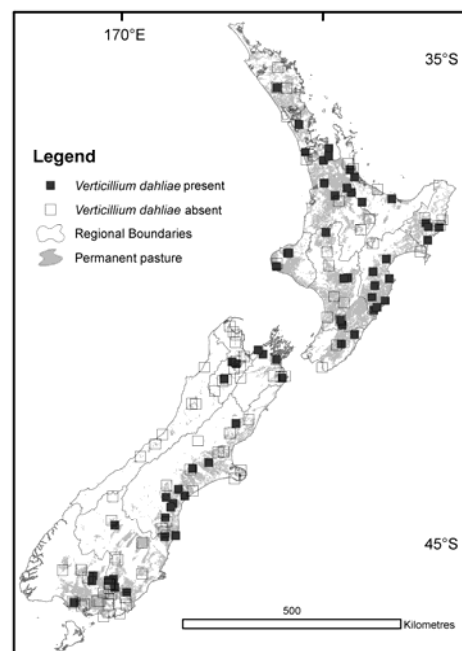
## Results and Discussion

Many different fungi were isolated from the symptomatic *C. arvense* samples (yellowing, leaf loss, wilting). One of these, *Verticillium dahliae*, occurred throughout the country and was present in the roots and/or stems of 54 (42%) of the 128 random farm samples and in 8 (40%) of the 20 extra samples (Map). Californian thistle is not reported as a host of *V. dahliae* in major international collections and databases but has occasionally been noted in publications (Leth & Haas, 1984; Waipara *et al.*, 1991). Its application to healthy potted *C. arvense* plants resulted in deaths, especially when applied to the roots as conidia or microsclerotia (Table). Browning occurred in the vascular tissues of roots of inoculated plants and *V. dahliae* was re-isolated from roots, stems, leaves and new shoots of symptomatic plants confirming it as the causative agent.

Percentage mortality of *C. arvense* plants 3 months after treatment with *V. dahliae* (conidia,  $10^6$ /ml; microsclerotia,  $10^3$ /ml). Means over 3 isolates and 5 plants/ isolate.

	Conidia	Micro sclerotia	Water
Root dip	87	47	0
Sprayed on cut shoots	33	13	0
Sprayed on intact shoots	0	0	0

The life history of *V. dahliae* suggests it could be responsible for the demise of *C. arvense* populations mown in the rain, (a phenomenon reported to us by two farmers in 2005 and four in 2008). It produces two types of dispersal bodies: (a) resistant microsclerotia which reside in the soil, initiate infection of new roots and add to the soil inoculum as roots decay, and (b) small asexual spores (conidia) produced on fungal strands within vascular tissues of infected roots. Conidia released into the xylem are carried upwards to colonise stems and leaves, where they cause the characteristic symptoms of wilting, leaf yellowing and stem death due to blockage of xylem vessels and from where they could readily be dispersed to other shoots in a *C. arvense* population during mowing under wet conditions. In field experiments funded by Meat & Wool NZ, we are currently testing the idea that mowing in the rain can effectively control *C. arvense* in pastures by dispersing the naturally-occurring *V. dahliae*.



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**Root Colonization and Environmental Fate of the Bioherbicide *Pseudomonas fluorescens* BRG100**

Susan M. Boyetchko, Caressa Hanson, Russell K. Hynes, and Darren Korber  
Agriculture and Agri-Food Canada | Agriculture et Agroalimentaire Canada  
Saskatoon, Saskatchewan S7N 0X2  
boyetchkos@agr.gc.ca

*Pseudomonas fluorescens* BRG100 is a bacterial strain which is being developed as a pre-emergent soil-applied bioherbicide for control of the annual grass weeds wild oat, *Avena fatua*, and green foxtail, *Setaria viridis*. The green fluorescence protein gene was introduced into *P. fluorescens* BRG100 from *E. coli*  $\lambda$ S17-1 carrying suicide vector pAG408 in order to permit the visualization and monitoring of root colonization and environmental fate when introduced into soils under various environmental conditions. Colony morphology, growth rate, weed biocontrol efficacy (plant growth pouch), carbon utilization (Biolog GN) and root colonization of green foxtail by several *P. fluorescens* BRG100*gfp* transformants were very similar to that of the wild type. *P. fluorescens* BRG*gfp*-15 was found to be most similar to the wild-type in all of the above characteristics and was chosen for subsequent experiments. It was determined by population dynamics per section of root from plating on culture medium, fluorescence microscopy and confocal microscopy that *P. fluorescens* BRG*gfp* colonized all areas of the root, but showed a preference for the proximal 1/3 section and the seed, when applied as a liquid inoculum or as the pesta granular formulation. Confocal microscopy revealed that the root hairs, root tip, and ventral portion of the seed were all areas of heavy root colonization. Survival of *P. fluorescens* BRG*gfp* was investigated by conducting a study using a thermogradient plate apparatus in two soil types under three temperature and three moisture levels typical of environmental conditions prevalent in the Canadian prairies during the early spring, summer and early fall. The experiment was set up as a factorial randomized complete block design that included the following treatments: three 12 hour diurnal temperature regimes: 5-15°C, 15-25°C, and 25-35°C and three moisture levels: 25, 50 and 75% of field capacity. Sampling was carried out at 0, 14, 28 and 42 days. The highest numbers of viable bacteria were found in the pesta granules in soil subjected to the lowest diurnal temperature regime and moisture content. The lowest numbers of viable bacteria were found in the pesta subjected to the highest diurnal temperature and moisture, suggesting the release and dissemination of BRG*gfp* from pesta granules is greater at higher temperatures and moisture levels.

## **Herbicide-Deleterious Rhizobacterial Interactions in Velvetleaf Weed Control**

Robert Zdor Dept. of Biology, Andrews University, Berrien Springs, MI 49104

\*zdor@andrews.edu

### **Abstract**

Previous work by other researchers has shown that herbicide treatment can increase plant vulnerability to microbes. The use of biological agents to augment herbicide-based weed control was examined in this study. Specifically two rhizobacterial isolates were studied: *Pseudomonas putida* ATH2-1RI/9 and *Acidovorax delafieldii* ATH2-2RS/1. These organisms were originally isolated from the roots of velvetleaf and show modest growth reductions in velvetleaf assays. In this current study, velvetleaf plants were grown in soil inoculated with either bacterium ( $10^9$  cells) in the presence or absence of sublethal levels of atrazine & metolachlor (0.28  $\mu$ g active ingredient/g soil). Plant survival and weight were scored after 4 weeks. Plant survival in the absence of herbicide was over 90% regardless of the presence of bacteria. Approximately 78% of the plants survived when treated with the herbicide alone. However dramatic decreases in plant survival were obtained in plants exposed to both herbicide and *A. delafieldii* ATH2-2RS/1. This synergy was not seen in plants exposed to herbicide and *P. putida* ATH2-1RI/9. Plants that survived the herbicide/*A. delafieldii* ATH2-2RS/1 treatment had shoot weights that were 53% of plants exposed to herbicide alone. This data suggests that certain microbes have the ability to act synergistically with herbicides opening the possibility for weed control strategies that involve reduced levels of herbicide. Further work with these particular microbes needs to be done in the field to confirm efficacy.

### **Introduction**

Velvetleaf (*Abutilon theophrasti*) roots are colonized by a variety of bacteria, some of which have plant growth-suppressing properties (Kremer et al. 1990). This finding has helped prompt research on deleterious rhizobacteria (DRB) as a possible component in velvetleaf control strategies. Two rhizobacteria (*Pseudomonas putida* ATH2-1RI/9 and *Acidovorax delafieldii* ATH2-2RS/1) recovered from velvetleaf roots reduce velvetleaf, but not corn, growth in autoclaved soil in growth chamber studies (Owen and Zdor 2001). Efforts to improve the performance of these organisms have turned to the possible use of herbicides as a synergistic agent. A variety of herbicides have been shown to influence rhizobacterial interactions with roots. Greaves and Sargent (1986) demonstrated that mecoprop treatment of wheat roots increased levels of fluorescent *Pseudomonas* spp. within cortical tissue presumably by damaging the roots and promoting root invasion. The root inhibiting herbicides pendimethalin and trifluralin reduced levels of inoculated *Pseudomonas fluorescens* in the cotton rhizosphere thereby impacting the effectiveness of these organisms in plant disease suppression (Heydari et al. 1997). In a study that parallels this current study, synergy was noted between sulfosate and a variety of proprietary bacteria in injuring a number of weeds, including velvetleaf (Christy et al. 1993). The nature of how sublethal levels of herbicide influence bacterial-weed interactions is unclear but work with fungi has implicated altered plant defense mechanisms as a possible explanation (Nickerson et al. 1993; Sharon et al. 1992). With the long-term goal of improving the performance of *P. putida* ATH2-1RI/9 and *A. delafieldii* ATH2-2RS/1 in suppressing weed growth this current study examined the effect of sublethal levels of atrazine/metolachlor herbicide on hindering velvetleaf growth and survival in the presence of these two DRB.

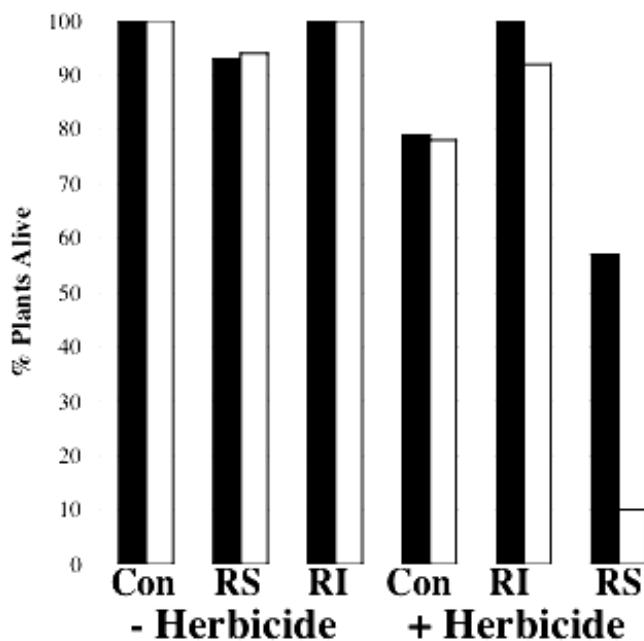
### **Materials and Methods**

Velvetleaf seeds were planted individually in Cone-tainers (Stuwe and Sons) using autoclaved soil (14-21 plants per treatment) in the presence or absence of

atrazine/metolachlor (0.28 µg active ingredient/ g soil; supplied as Bicep<sup>TM</sup>), *P. putida* ATH2-1RI/9, or *A. delafieldii* ATH2-2RS/1 (10<sup>9</sup> cells/plant). After four weeks of growth in a growth chamber, the number of surviving plants determined along with plant dry weight. The experiment was repeated twice.

## Results and Discussion

Seedling emergence for all 6 treatments was 100%. Plants growing in the absence of herbicide had excellent survival with over 90% of the plants alive at 4 weeks regardless of the presence of bacteria (Figure 1). Seventy eight percent of plants growing in the presence of herbicide and the absence of bacteria survived. Interestingly plants exposed to both herbicide and *P. putida* ATH2-1RI/9 had a best survival of all herbicide-treated plants. More significantly, synergy was seen between atrazine/metolachlor and *A. delafieldii* ATH2-2RS/1 in reducing plant survival with 33 percent (on the average) of plants surviving. The plants that did survive in this treatment had a shoot dry weight that was 53% of herbicide-treated plants lacking bacterial inoculation. Previous work examining synergy between sulfosate and various bacterial strains revealed strain-strain differences in velvetleaf injury (Christy et al. 1993). Preliminary results suggest that this synergy also occurs between *A. delafieldii* ATH2-2RS/1 and treatments of atrazine or Banvel<sup>TM</sup> (Han and Zdor, unpublished data). Further study is needed to examine if the presence of sublethal levels of herbicide promote root colonization of *A. delafieldii* ATH2-2RS/1. The results obtained are promising but efficacy must be evaluated in the field for the use of herbicide/DRB combinations to be a viable component of velvetleaf control strategies.



**Figure 1.** Velvetleaf survival grown in the presence or absence of Bicep<sup>TM</sup> herbicide with or without *P. putida* ATH2-1RI/9 (“RI”) and *A. delafieldii* ATH2-2RS/1 (“RS”). Plants were evaluated after four weeks of growth. Each bar represents the results from 14-21 plants. Solid bars: Experiment I. Open bars: Experiment II. Con: no bacteria

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## Using Gene Fusions to Study Cyanogenesis in a Weed Deleterious Rhizobacterium

M. M. Biswas\*, R. Zdor\*, C. Miller\*\*

\*Dept. of Biology, Andrews University, Berrien Springs, MI; \*\* Dept. of Biological & Irrigation Engineering, Utah State University, Logan, UT \*biswasm@andrews.edu

### Abstract

Previous work with weed deleterious rhizobacteria (WDR) has implicated the production of hydrogen cyanide in the ability to suppress plant growth. This current study examines factors that influence cyanogenesis in *Pseudomonas putida* ATH2-IRI/9, a WDR that colonizes velvetleaf roots resulting in cyanide in the rhizosphere and reduced plant growth (Owen and Zdor, 2001). The effects of oxygen and iron on cyanogenesis in *P. putida* ATH2-IRI/9 pure cultures were tested using 2 techniques. In the first method cyanide recovered from both early and late stage cultures growing in the presence/absence of iron &/or oxygen was quantified using a colorimetric assay. Secondly a gene fusion method was used as a reporter of cyanide production under the same culture conditions. In this method a promoterless luciferase gene (*luxAB*) (Miller et al. 1997) is inserted into the *hcnABC* operon of *P. putida* ATH2-IRI/9, which encodes HCN synthase, the enzyme responsible for hydrogen cyanide production. Culture conditions that influence expression of the *hcnABC* operon and thus cyanogenesis are then studied by measuring light production by the gene fusion-bearing bacteria in culture. The results from both techniques consistently showed that the presence of iron (under microaerophilic conditions) for both early and late growth stage cultures was the main variable that stimulated cyanogenesis resulting in cyanide and light levels approximately 5 times greater than levels from cells grown in the absence of iron. These results support similar findings obtained with the plant-associated bacterium *Pseudomonas fluorescens* CHA0 (Blumer and Haas 2000). Further work must be done to test the effect of iron in promoting cyanogenesis in the rhizosphere and its possible role in optimizing weed control strategies that involve cyanogenic WDR.

### Introduction

Velvetleaf (*Abutilon theophrasti*) is a very competitive plant that is a major weed problem in crops grown in the United States. *Pseudomonas putida* ATH2-IRI/9 has the ability to suppress weed seedling growth of velvetleaf and hydrogen cyanide production in the rhizosphere (cyanogenesis) is implicated in this effect on plant growth. Introduction of *P. putida* ATH2-IRI/9 into the rhizosphere may therefore be a way to improve agricultural production in an environmentally acceptable way. However knowledge of the factors that may affect their growth and survival, and their genetic and physiological responses to these conditions, is scarce.

After introduction to soil, several environmental conditions can influence the production of cyanide in the microorganism. The operon *hcnABC* constitutively expresses HCN synthase, an enzyme which catalyzes glycine to form carbon dioxide and hydrogen cyanide. The biosynthesis of the secondary metabolite hydrogen cyanide (HCN) is maximal during the transition from exponential to stationary phase and is influenced by several environmental factors including iron, phosphate and oxygen limiting conditions (Blumer and Haas 2000). Work from Blumer and Haas (2000) shows that the anaerobic regulator ANR acts as an iron sensor in the *hcn* operon which converts to its active form under low oxygen supply (Castric, 1983; Castric, 1994). To understand how *hcnABC* gene is being expressed in varying soil and culture conditions, we used a luminescence-based marker system which may be easily tracked through detection of light emitted by marked cells, without the requirement of cell extraction. Light (590 nm) is produced from the action of a luciferase enzyme by oxidizing

the substrate (tetradecanal) and reducing flavin (Prosser, 1996). We engineered fusions of promoterless luciferase structural genes (*luxAB*) to the *hcnABC* operon of *P. putida* ATH2-IRI/9. Bacterial luciferase genes are widely used as reporters of gene expression because of the high sensitivity of chemiluminescence detection and the possibility of monitoring light production in intact cells (Gonzalez-Flecha and Demple 1994). This study will identify how environmental conditions like iron and oxygen, influence cyanide production in the bacterium *P. putida* ATH2-IRI/9 using *hcnABC::luxAB* gene fusion in culture and soil.

### Materials & Methods

*P. putida* ATH2-IRI/9 carrying the *hcnABC::luxAB* gene fusion was grown in half strength King's B Broth overnight at 28°C and 100 µl of this inoculum was then subcultured onto a 250 ml erlenmeyer flask containing 100 ml of a synthetic glycine minimal medium (MMC) with and without 20 µM FeCl<sub>3</sub>, gentle shaking at 28°C in a water bath (Castric, 1975). Oxygen limitation was achieved by sealing the flask with a rubber stopper. After 18 and 30 hours of incubation at 28°C in a water bath, culture aliquots were aseptically removed to measure absorbance at 600 nm and bioluminescence in a Luminometer (Turner Designs 20/20 model). Light production was recorded for 2 minutes cumulatively. Dilution plating was used to determine CFU/ml of each culture. The same culture conditions were used to grow the wild-type *P. putida* ATH2-IRI/9 organism. HCN was quantified using the method of Lambert et al. (1975). All cultures were grown in triplicate and the experiment replicated three times.

### Results & Discussion

Bioluminescence of *P. putida* ATH2-IRI/9 cultures containing the gene fusion was strongest in the presence of iron (Figure 1A). Iron-containing cultures in stationary phase (30 hours) had the highest levels of light production. Interestingly microaerophilic conditions reduced expression of the *hcnABC* operon in the presence of iron. Using the wild-type organism, cyanide production increased about 1.5 times with Iron and oxygen limitation in the 18 hour culture sample while increases 5 times in the same group of 30 hour culture sample (Figure 1B). Cyanide production decreases as oxygen is maximum and iron depleted from culture in the 18 and 30 hour sample. There is a significant increase when oxygen limitation is achieved in the culture sample depleted with Iron but the increase is not as much as it is in the oxygen limitation group with Iron in sample. The results from both techniques consistently showed that the presence of iron (under microaerophilic conditions) for both early and late growth stage cultures was the main variable that stimulated cyanogenesis resulting in cyanide and light levels greater than levels from cells grown in the absence of iron. These results support similar findings obtained with the plant-associated bacterium *Pseudomonas fluorescens* CHA0 (Blumer & Haas, 2000). Further work must be done to test the effect of iron in promoting cyanogenesis in the rhizosphere and its possible role in optimizing weed control strategies that involve cyanogenic WDR.

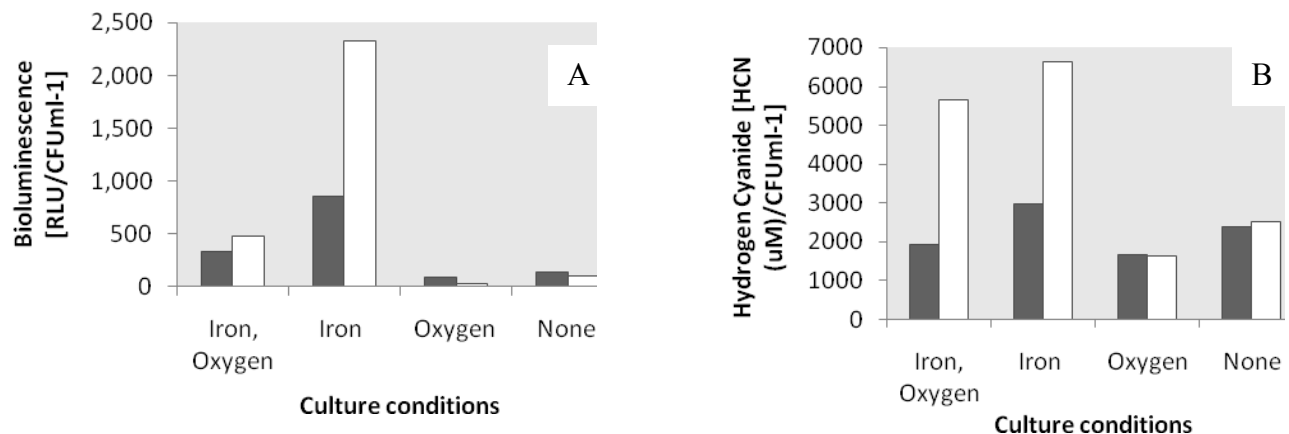


Figure 1 Bioluminescence activity (A) and Hydrogen cyanide production (B) of *Pseudomonas putida* ATH2-IRI/9. Mean values from three different experiments are presented. Grey bars, 18 hour sample; White bars, 30 hour sample. Iron: iron-supplemented culture; oxygen: non-microaerophilic culture. RLU: relative light units.

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### **Evaluation of *Alternaria alternata* as a potential biocontrol agent for field bindweed (*Convolvulus arvensis*)**

Ehsanallah Zeydali, Natural Research Center of Lorestan, Iran; Alireza Koocheki, Ferdowsi Univ. of Mashhad, Iran; Nader Azadbazht, Natural Research Center of Lorestan, Iran and Reza Ghorbani\*, Ferdowsi Univ. of Mashhad, Iran. \* Email: ghorbani43@gmail.com

#### **Abstract**

Field bindweed can be found in a wide range of habitats and causes substantial yield reduction in many crops. *Alternaria* spp. are airborne molds that were proposed to have potential for the biological control of weeds. Results of the present study showed that *Alternaria alternata* isolate A2 developed the most infection Among different spore concentrations ( $10^4$ ,  $10^5$ ,  $10^6$  and  $10^7$  spores in 1ml distilled water) the treatment of  $10^7$  spores in 1ml at 4-leaf stage caused most weed control of field bindweed. The maximum disease development were observed with application of the fungus of *A. alternata* at dew periods of 24 and 48 hour, however, plant damage was also observed with a length of 6 hours dew period. These experiments have confirmed the potential of *A. alternata* as a mycoherbicide under specific environmental conditions.

**Key words:** Biological control, Bioherbicide, Dew period, Saturation humidity, Spore concentration.

#### **Introduction**

Field bindweed (*Convolvulus arvensis*) is an important perinial weed of agricultural crops world-wide that can be found in a wide range of habitats and causes substantial yield reduction and causes problems in harvest in many crop. Biological control agents, especially plant pathogenic fungi, have been shown to have control potential for specific problem weeds. However, under field conditions control activity of mycoherbicides is often variable. Detailed knowledge about the environmental conditions required for disease development and weed mortality is an important prerequisite for the development of mycoherbicides and their use in integrated weed control strategies (Ghorbani et al, 2005). Susceptibility of plants to a given dose of pathogen inoculum may vary with weed growth stage. In some plant pathogen combinations, the hosts are susceptible only in seedling stages, and older plants become resistant (Agrios, 1997), although the pattern varies depending on pathogen host systems. Kadir and charudatton (2000) reported which *Cyperus rotundus* in 4- 6 leaf stage is more sensitive toward older plant (8 leaf stage), to fungus *Dactylaria higginsii*. Ghorbani et. al (2000) found that the best stage for biological control of *Amaranthus retroflexus* with fungus *Alternaria alternata* observed at 2-4 leaf growth stage. . Abundant, prolonged, or repeated high moisture, whether in the form of rain, dew, or high humidity, is the dominant factor in the development of most epidemics caused by fungi, bacteria and nematodes (Greaves et al. 1999). The aim of this study was to investigate the effects of field bindweed developmental stage, spore concentration, temperature and humidity or dew period on the activity of different *Alternaria alternata* to assess their potential for biological control.

#### **Material and Methods**

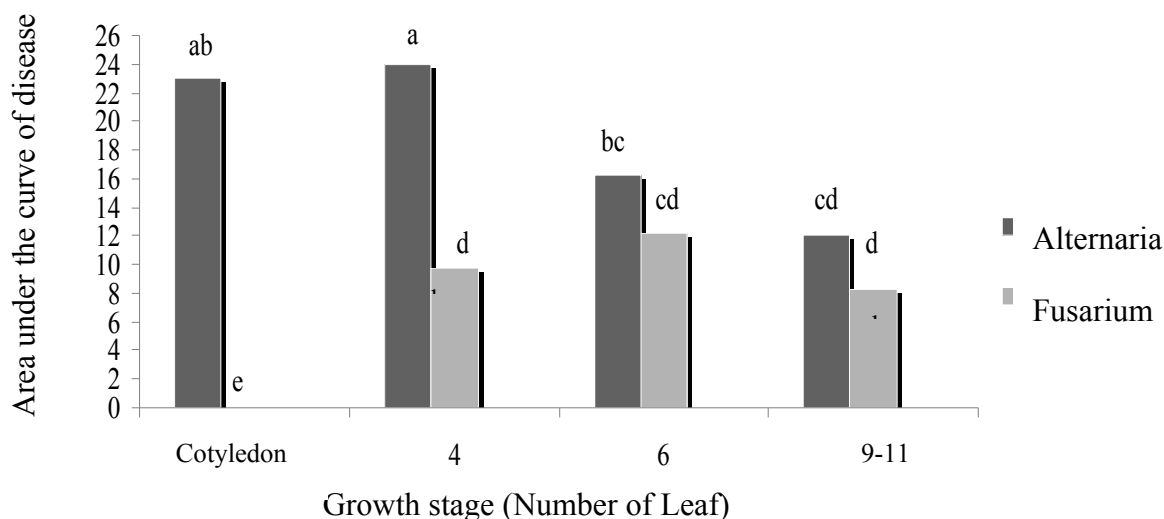
A series of studies were carried out in Faculty of agriculture, Ferdowsi University of Mashhad, and Natural Research Center of Lorestan, Iran, in order to evaluate *Alternaria alternata* collected from Mashhad, against field bindweed. The weed seeds were planted in the pots with 10cm diameter and 15cm height contains sandy loam soil. In the first experiment, three plants per pot at different growth stages (cotyledons, 4, 6 and 9- 11 leaf-stages) were sprayed with  $10^7$  spores per ml of *Alternaria alternata* strain A2. In the

second experiment, three *Convolvulus arvensis* plants per pot were sprayed with a suspension containing different *A. alternata* spore concentrations of  $10^4$ ,  $10^5$ ,  $10^6$  and  $10^7$  spores in 1ml distilled water. Control plants were sprayed by distilled water. To create a relative humidity of over 90%, treated plants were immediately covered with plastic bags for 48 h. Pots were then placed in a greenhouse with 60 to 65 %relative humidity. Three plants per pot were scored for disease development 3 and 10 days after spraying using the following scoring system: 0= no symptoms, 1= 1to 25% necrosis of total leaf area, 2= 26 to 50% necrosis of total leaf area, 3= 51 to 75% necrosis of total leaf area, 4= 76 to 99% necrosis of total leaf area, 5= %100 necrosis of total leaf area (plant death). After the second assessment, plants were cut at the soil surface and their aboveground fresh weight was determined. Plant materials were dried in an oven (70 C, 48 h) and dry weight was recorded. For dew requirement experiments, *C. arvensis* plants at 2-4 leaf stage were sprayed with suspensions of  $10^7$  spores per ml of *A. alternata*. Treated plants were covered with plastic bags to maintain %90 humidity and incubated for 6 ,12, 24 or 48 hours in growth cabinets.

### Results and discussion

Results showed that *Alternaria alternata* isolate A<sub>2</sub> had a higher effects than the fungus *Fusarium sp.*. The effect of fungi was significantly different ( $p < \%5$ ) between plant in different growth stages and both fungi were more effective at 2-4 leaf-stages (Fig. 1).

Fig. 1. Interaction effects of plant growth stage and fungi isolates of *Alternaria sp.* and *Fusarium sp.* On area under the curve of disease development (AUCDD) in field bindweed. Different letters indicating differences between means based on Duncan test (%5).



The maximum disease development were observed with application of the fungus of *A. alternata* at dew periods of 24 and 48 hour, however, plant damage was also observed with a length of 6 hours dew period (Figs 2 and 3).

Fig. 2- Effect of dew period length on disease development caused by *A. alternata* in field bindweed. Different letters indicating differences between means based on Duncan test (%5).

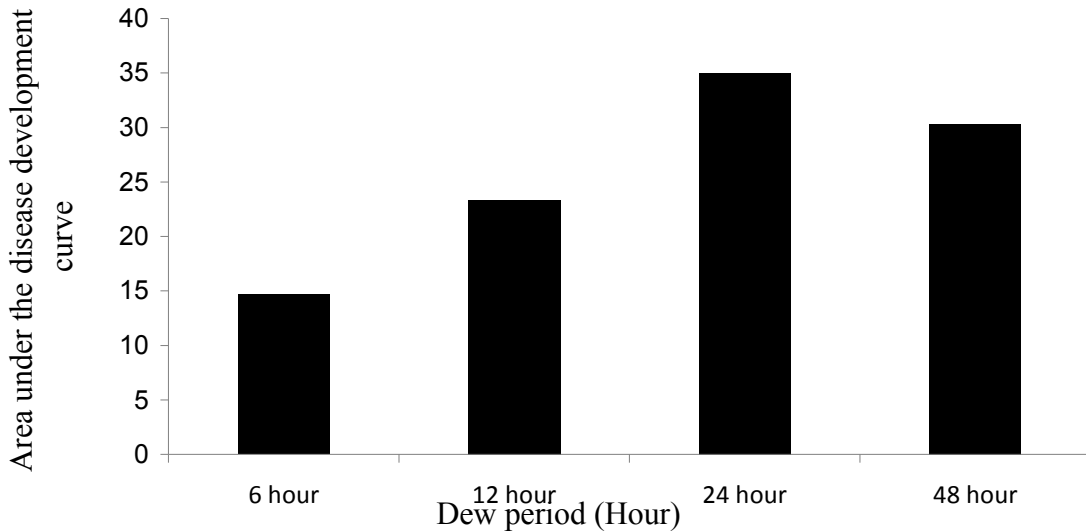
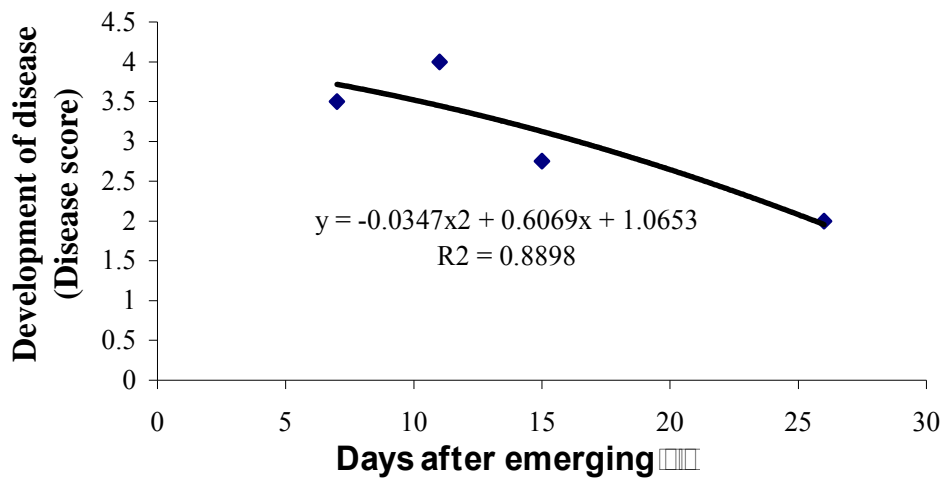


Fig. 3- Effect of dew period length on the area under the curve of disease development (AUCDD) OF *Alternaria alternata* in field bindweed of 4 leaf stage.



In conclusion, for maximum activity of *A. alternata* as a mycoherbicide for *C. arvensis*, the inoculum should be applied (a) at a rate of  $10^7$  spores ml, (b) to plants at the two- to four-leaf growth stages, and (c) when environmental conditions are favorable for high humidity to occur on the foliage of *C. convolvulus*, for at least 12 hours after inoculation. According to the above findings, there are two major limitations for using *A. alternata* as a mycoherbicide.

First, the need for high spore density may increase costs of application in commercial practice. Second, the long dew period (24 hours) required to maximize activities may not be achieved in many climatic zones in the absence of irrigation. Further research is therefore needed to decrease the requirement for high spore density and long dew period. Research should focus on improving spore formulation and isolation of more effective *A. alternata* strains.

### **Acknowledgment**

The authors gratefully acknowledge the Faculty of Agriculture, Ferdowsi University of Mashhad, Iran for financial supports.

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**Fungal toxins and other natural metabolites for management of parasitic weeds**

Maurizio Vurro & Angela Boar, Istituto di Scienze delle Produzioni Alimentari – CNR via Amendola 122/O – 70125, Bari, Italy

Difficulties in controlling parasitic weeds are due to their physiological traits, i.e., among others: the production of large amounts of seeds, that can remain viable for many years; the germination of seeds, that occurs only by stimulation with host root exudates; the production of a germ tube, that develops a haustorium penetrating the root and forms a tubercle if it attaches to the host root; the long underground damaging phase, with the parasitic withdrawal of water, nutrients and photosynthates from the host. Preventing seed germination and host attachment could be a strategic and successful interference with the parasite for its management. Natural compounds which are able to inhibit seed germination or, on the opposite, to stimulate it in absence of the host plant, could be attractive and environmentally friendly tools to reach that objective. Our research was focused in particular on: a) fungal toxins having inhibitory activity on seed germination; b) amino acids inhibiting seed germination and tubercle development; and c) fungal metabolites stimulating the "suicidal" germination. The target parasitic weed was mainly *Orobancha ramosa* L. (commonly named broomrape), a very aggressive parasite of many crop species, and to a lesser extend *Striga hermonthica* (Del.) Benth (witchweed) and *Cuscuta campestris* Yuncker (dodder), two other very dangerous parasitic weeds. With regard to the use of fungal metabolites, several compounds were assayed choosing among: 1) already known microbial compounds produced by *Fusarium* species and available commercially or at laboratorial level; 2) compounds obtained from the cultures of potential biocontrol agents of *O. ramosa*; 3) novel phytotoxins recently isolated and chemically characterized in our labs from the cultures of fungi pathogens of weeds other than parasitic ones. With regard to the possible use of amino acids, this approach was based on the mechanism of action of the so called "Frenching disease", a physiological disorder of tobacco caused by saprophytic bacteria growing on the roots, overproducing isoleucine. Shortly, the idea was to supply high or unbalanced amounts of essential amino acids to cause disorders in the metabolic processes that occur during the germination of seeds, then inhibiting the process of germination or the elongation of the germ tube. This is the same final results of some chemical herbicides that inhibit single biosynthetic enzymes in plants, making treated plants incapable of producing metabolites essential for plant growth. The promising results obtained by using both fungal metabolites and natural amino acids will be shown and discussed.

## Pathogenic Mechanisms of Vulclic Acid to Alligatorweed

Xiang, M. M.<sup>1\*</sup>; Fan, L. L.<sup>1</sup>; Jiang, Z. D.<sup>2</sup>

<sup>1</sup> Agriculture and Landscape College, Zhongkai University of Agriculture and Engineering, Zhongkai Road, Guangzhou 510225, China

\* zyp@zhku.edu.cn; mm\_xiang@163.com

<sup>2</sup> Resource and Environment College, South China Agricultural University, Wushan, Guangzhou 510642, China

### Introduction

Kimura *et al.* (1991) isolated vulclic acid from the filtrate of *Penicillium* sp. and found its inhibition to the pollen germination of *Pinus thunbergii*. Zhou *et al.* (2006) also obtained the toxin from the filtrate of *Nimbya alternantherae* and Xiang *et al.* (2008) proved it could cause leaf blight and withering of alligator weed. The preliminary result indicated that the damage of the toxin on the ultrastructure of leaf tissue included plasmolysis and vacuolation in cells, lamellae disorder and vacuolation in chloroplasts as well as disappearance of ridges and vacuolation in mitochondria (Xiang *et al.*, 2008). In order to prove the pathogenic mechanisms of vulclic acid and establish a foundation for the development of it, we carried out a further study on the effect of the toxin on ultrastructure of leaf and root tip tissues of alligator weed.

### Materials and methods

The purified vulclic acid was diluted to concentrations of 50 and 100 mg/ml with double-distilled water. The little plants of alligator weed from the field were cultured in the nutrient solution till 5~6 pairs of new leaves coming out. Healthy plants were washed with tap water and then three times with double-distilled water, dried on sterile filter paper. The plants were transplanted into tubes containing 10 ml of the different toxin dilutions. The mature leaves were cut into pieces of 2 to 3 mm transversely, placed into test tubes with 2 ml of the different toxin solution and decompressed for 20 min. Then, the tubes with solution and plants or leaf pieces were put into an illuminated incubator at 25°C for 12 and 24h, respectively. Double-distilled water was used in the control tube. After that, root tips of 2mm long were cut off as samples.

The samples were fixed with 2.5% glutaraldehyde, then with 1% osmic acid. After being dehydrated using a standard method, the samples were embedded in Epon 812. Microtome sections were dyed with uranium acetate, then lead citrate and observed through FEI-Tecna 12 transmission electron microscope.

### Results and discussion

The results showed that the damages were disruption of plasma membrane, digestion of the membrane of chloroplast and mitochondria, disorder of chloroplast lamellae and digestion of mitochondria ridge in leaf tissue, and disruption of plasma membrane, digestion of mitochondria membrane and ridge and vacuolation and shrinking of meristematic and epidermal cells in root tip tissue. The severity of damages was increased along with the increasing of toxin concentration and treated time. The lamellae of chloroplast swelled and mitochondria vacuolated then membrane disruption appeared in both of chloroplast and mitochondria at the lower concentration (50µg/mL), while disruption of membrane and swelling of chloroplast lamellae and digestion of mitochondria ridge occurred at the same time or the former was prior to the latter two at the higher concentration (100µg/mL). The ultrastructure changes of treated root tip tissue with the toxin were similar. These results suggest that the initial sites for the toxin action may be on chloroplast and mitochondria.

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**Surfactants affect the efficacy of *Alternaria cassiae* controlling sicklepod seedlings**

R. A. Pitelli\*, C. F. Franco, F. M. Claudia; University of State of Sao Paulo, Jaboticabal, Brazil

A Brazilian isolate of *Alternaria cassiae* was collected in Brasília, Brazil, from seedlings showing symptoms of necrotic lesions on leaves and petioles. Several studies done in Brazil and USA showed that this fungus has very good potential as bioherbicide candidate. For field applications, some spraying technology development is necessary. So, the objective of this research was to evaluate (i) the effects of the surfactant Energic (0.1 and 0.2%), Agral (0.025 and 0.05%), Herbitensil ((0.1 and 0.2%) and Citowett (0.1 and 0.2%) on *A. cassiae* conidia germination and (ii) the effects of spores concentrations ( $10^4$  and  $10^5$  spores/mL), surfactants (same products and concentrations) and dew period (0, 6, 12 and 24 hours) on disease development and control of sicklepod plants. The spore suspension was disposed over water-agar media enriched with the surfactants treatments. Conidia germination was not affected by surfactants at the tested doses. The effects of surfactants only were significant in the lower spore concentration. Citowett and Agral promoted faster disease development when compared with the other two surfactants. Energic (0,2%) reduced the sicklepod control sprayed with  $10^4$  spores/mL. Dew periods of 8, 12 and 24 hours promoted 100% plant mortality while the absence of artificial induced dew period the sicklepod control was only 20%.



### **Potential of *Cassida rubiginosa* as a biological control agent for *Cirsium arvense***

Ghorbanali Asadi, Reza Ghorbani, Mohammad-Hassan Rashed-Mohassel\* and Hossein Sadeghi, Department of Agronomy, Faculty of Agriculture, Ferdowsi University of Mashhad. P. O. BOX 91775-1163, Mashhad. Iran. Email: mhrashed@yahoo.com

#### **Abstract**

Canada thistle is one of serious weeds throughout the world and third serious weed in Europe. The control of Canada thistle is very difficult due to its extensive lateral and diffused root systems, the lignified and spiny form of its stem and leaves and also the ability of high seed production. Canada thistle seriously decreases crops yield. This study as started from 2006 and so far 12 branches arthropod contain a mite herbivore belonging to Arachnida and 12 species belonging to Coleoptera have been found. Among these agents, *Cassida rubiginosa* species showed high population and efficiency on Canada thistle and therefore this species can be a promising biological control agent for Canada thistle.

Keywords: Biocontrol insects, *Cassida rubiginosa*, Herbivores, Natural enemies.

#### **Introduction**

Canada thistle (*Cirsium arvense* (L.) Scop.) is a rhizomatous perennial thistle, 0.5 - 1.0 m tall, distinguished from other thistles by (i) creeping horizontal lateral roots; (ii) dense clonal growth; and (iii) small dioecious (male and female flowers on separate plants) flowerheads. The concern about herbicide residues in foods and the environment, along with increasing incidence of herbicide resistance in weeds, have resulted in the need for alternative approaches of weed control methods. Biocontrol agents offer possible alternatives to chemical herbicides for the control of problematic weeds as a practical, safe, environmentally beneficial, weed management method applicable to agroecosystems. Canada thistle is considered as one of the world's worst weeds and the third troublesome weed in Europe. causes extensive crop yield losses through competition and, perhaps, allelopathy (Stachon and Zimdahl, 1980). It has become increasingly problematic in ecological compensation areas where conventional control measures are restricted. Thus, biological or integrated control, exploiting both plant competition and herbivory by specialized native insects, may be an inexpensive and sustainable alternative control measure. There has been little study of the impact of herbivory on natural populations of this weed. Making use of native agents for weed biocontrol requires increasing natural enemies. To date, augmentation or conservation of native agents received little attention compared to other approaches but interest in the use of native agents is growing. Future progress in classical biological control of *Cirsium arvense* will depend on the identification of new, adequately host specific herbivores from its native range, and will require improvements in host-testing procedures to allow better prediction and evaluation of non-target impacts.

To date, augmentation or conservation of native agents has received little attention compared to other approaches but interest is growing. Making use of native agents for weed biocontrol requires to increase naturally occurring population densities. To date, augmentation or conservation of native agents received little attention compared to other approaches (Julien 1998; McFadyen. 1998; Müller-Schärer 2000), but interest in the use of native agents is growing (Müller-Schärer et al. 2000). In a number of cases, native biocontrol agents were able to successfully control their target weed (Julien 1998). The green thistle beetle (*Cassida rubiginosa* Müller (Coleoptera: Chrysomelidae)) is a chrysomelid foliage-feeding beetle that has a wide Palearctic (Europe, North Africa, northern Asia) distribution. Unfortunately, successes are poorly documented. Therefore, experienced strategies for biocontrol attempts involving native agents are lacking.

### **Material and Methods**

Different parts of *Cirsium arvense* at different sites of North Khorasan are being surveyed for the presence of insect herbivores. Various arthropods contain a mite herbivore belongs to Arachnida and 12 species belong to Coleoptera order have been found. All of collected herbivores were studied in several leaf bioassay and field studies. The effects of number of insect per plant, and also effects of *C. rubiginosa* on competition between Canadian thistle and wheat in the field were studied. Safety tests were carried out in the greenhouse experiments on a wide range of crops including wheat, sunflower, safflower, maize, canola, sugar-beet, spinach, chickpea, bean, soybean, egg-plant, tomato, potato, green pepper, cucumber, melon, water melon and cotton.

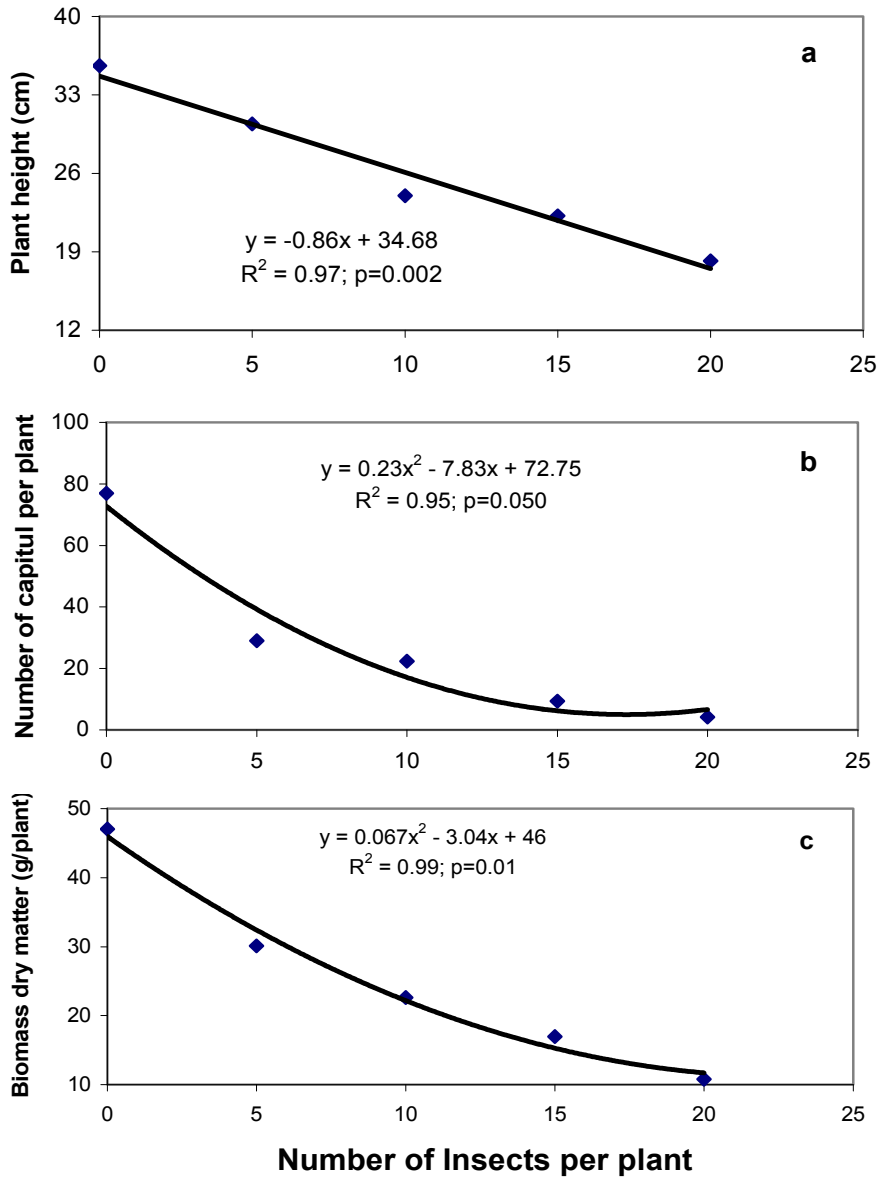
### **Results and Discussion**

It is realized that *Cassida rubiginosa* a univoltine shield beetle feeds on foliage of *Cirsium arvense*, both as adults and larvae in North Khorasan, Iran. Defoliation by *C. rubiginosa* was most effective at high insect density on young plants. Among different collected herbivores, *Cassida rubiginosa* species showed the highest population and efficiency on Canada thistle. In field surveys, consistent adult and larval feeding were observed on *Cirsium arvense*. The insect of *Cassida rubiginosa* could significantly decrease the plant height, flowering and biomass dry weight of *Cirsium arvense* (Fig. 1). At a density of 20 beetles/plant, over 70% of the thistles died by the end of the growing season. *Cassida* impact was substantially reduced competition of Canadian thistle in the wheat fields. Results of safety tests showed that there was no any damage on tested crops except only about 20% damage caused by the insect of *C. rubiginosa* on safflower plants.

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Fig 1. Effects of *Cassida rubiginosa* on plant height, flowering and biomass dry weight of *Cirsium arvense*



**Native Phytopathogens as Biocontrol Agents: Problems and Potential in the Management of Invasive Exotic Species**

K. Jayachandran and K. G. Shetty; Florida International University, Miami, FL

The fragile natural ecosystems in Florida are being threatened by exotic invasive plant species. In addition to the environmental cost there is a huge economic cost associated with the expanding threat of exotic invasive plant species. Biological control using natural enemies is one of the environmentally compatible tools that are being used along with other control methods for the management of invasive species. Biological control of exotic invasive plant species has been predominantly oriented towards re-association of co-evolved natural enemies by introduction of selected natural enemies primarily insects from different countries. Introduction of another non-native biological agent into an ecosystem is fraught with unknown risks. At present many of the natural and agricultural ecosystems in Florida are being invaded by a long list of exotic plant species. The situation calls for the development of new capabilities to counter this growing threat. One option is to discover and develop additional biocontrol agents that can be integrated with the existing control methods. Native plant pathogen induced diseases on exotic plant species is not uncommon. Using native pathogens for biocontrol does not involve the introduction of any new organism and may also prove to be valuable for integrating with other control methods. Surveys of *Lygodium microphyllum* (old world climbing fern) and *Schinus terebinthifolius* (Brazilian pepper) infested sites in South Florida indicated occurrence of diseases caused by native plant pathogens. However, there are considerable challenges to be overcome before further development and deployment of effective native biocontrol agents can be made. Isolated and scattered efforts will have limited success, in order to fully realize the benefits of native pathogens, a collaborative and coordinated multi-institution/agency effort is very critical.

**Keyword** : Native Pathogens ; Exotic Invasive ; Biocontrol.

## **Media studies on *Myrothecium roridum* Tode; a potential biocontrol agent for water hyacinth**

Okunowo, W.O.<sup>1\*</sup>, Gbenle, G.O.<sup>1</sup>; Osuntoki, A.A.<sup>1</sup>; Adekunle, A. A.<sup>2</sup> <sup>1</sup>Department of Biochemistry, College of Medicine, University of Lagos, Lagos State, Nigeria. <sup>2</sup>Botany and Microbiology Department, University of Lagos, Akoka, Lagos State, Nigeria  
\*modelprof@yahoo.com.

### **Abstract**

Water hyacinth (*Eichhornia crassipes*) is a noxious aquatic weed in Nigeria and many parts of the world. A potential mycoherbicidal agent for the control has been identified recently as *Myrothecium roridum* Tode. The best media for in-vitro propagation was investigated using eight culture media; potato dextrose agar (PDA), tap water agar (TWA), Malt extract agar (MEA), potato sucrose agar (PSA), sabouraud agar (SA), potato carrot agar (PCA), Czapek-Dox agar (ZA) and a semi artificial diet, which included the material from the fungal host's plant (WHA). The mycelia growth was assessed by diameter measurement on agar plates and the conidial yield was measured with a Neubauer haemocytometer slide. The mycelia growth was maximum on TWA and minimum on ZA. The conidial yield was highest on MEA. Also, the mycelia growth and spore concentration of the fungus were highest on sodium glutamate and glutamine respectively, when used as a nitrogen sources.

### **Introduction**

Water hyacinth (*Eichhornia crassipes*) is a noxious aquatic weed in Nigeria and many parts of the world. Conventional management approaches includes; manual, mechanical and chemical control. These approaches have been found inadequate and expensive. Globally, the current trend in the management involves the use of biocontrol agents. A potential mycoherbicidal agent for the control has been identified in a recent study as *Myrothecium roridum* Tode (Okunowo et al., 2008a). The aim of this work is to develop a low cost media for the in-vitro propagation of the isolate.

### **Materials and methods**

Eight culture media were used to access the mycelia and conidial growth of the fungus; *Myrothecium roridum* Tode (IMI394934) isolated and characterized in a previous study (Okunowo et al., 2008a,b). The media included: PCA medium (potato 20 g, carrot 20 g and agar 20 g), PDA medium (diced potato 200 g, dextrose 15 g and agar 20 g), SA medium (glucose 40 g, peptone 10 g and agar 15 g), TWA medium (agar 18 g), WHA medium (dried powdered water hyacinth leaf 50 g, agar 18 g and fresh hyacinth leaf extract {100 g/litre of distilled water}) and ZA medium (sodium nitrate 2 g, potassium nitrate 1g, potassium chloride 0.5 g, magnesium sulphate 0.5 g, ferrous sulphate 0.01 g, sucrose 30 g and agar 20 g). The media also included; MEA (malt extract, 20 g, agar 20 g) and PSA (potato, 200g; sucrose, 20 g). Each culture medium was prepared in 1litre of distilled water and autoclaved at 120°C at 15 psi for 20 min. Two bisecting lines were drawn on the lower part of a sterile Petri dish. The prepared medium (10 ml) was added into the sterile Petri dish and allowed to solidify. A mycelia plug of the isolate was cut with a cork borer and placed in the centre of the Petri dish at the point of bisection. This was incubated at 25 ± 2°C. The diameter growth of the isolate was measured along the two bisecting lines. And the average diameter measurement was recorded. At the end, fourteenth day of the growth study, the spore concentration was estimated for each medium using a Neubauer haemocytometer slide. This was done by adding 1 ml of sterile distilled water (containing 0.1% v/v Tween 80 solution) into the Petri dish. The spore suspension obtained was diluted as appropriate. A drop of the suspension was made onto a Neubauer haemocytometer slide and the spores

were estimated as: Spore concentration (Spores/ml) = Number of spores counted/Number of area counted X Dilution factor X 10<sup>6</sup>. To determine the effect of nitrogen sources on microbial growth, Czapek Dox agar medium (sodium nitrate 2g, potassium nitrate 1g, potassium chloride 0.5g, magnesium sulphate 0.5g, ferrous sulphate 0.01g, sucrose 30g and agar 20g) was prepared such that it contains one of the following as nitrogen source (2g/litre): ammonium chloride, ammonium nitrate, glutamine, sodium glutamate and sodium nitrate. These were also autoclaved, inoculated and used for growth measurement as above.

### **Results and discussion**

All the media supported the growth of the fungus at varying degree (Fig. 1). The mycelia growth on day 14 was maximum on TWA and minimum on ZA (Fig. 1). The growth rate of the organisms was determined on all the media types as colony size per day. The result obtained showed that the organism had a different growth rate on each medium (Table 1). The highest growth rate was recorded on TWA, and this was significantly greater than that obtained in other the media types ( $P < 0.05$ ). It should also be noted that water hyacinth leaf agar (WHA) also supported the mycelia growth of the organism, and that WHA and TWA were the cheapest media used in this study. This also suggests that the mass production of the organism using the formulated water hyacinth leaf agar (WHA) medium may be economical. Sporulation of *M. roridum* was best on MEA followed by PSA and the least was observed on TWA (Table 2). The spore concentration of this isolate on MEA was significantly greater than those of other media types used ( $P < 0.001$ ). Also, the mycelia growth of the fungus was maximum on sodium glutamate and least on ammonium chloride (Fig. 2). While the highest spore concentration was recorded on glutamine as a nitrogen source (Table 3).

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OKUNOWO, W.O, Gbenle, G.O, Osuntoki, A.A. & Adegunle, A. A. 2008b. Investigative study of *M. roridum* toxin on water hyacinth. *Phytopathology* 98: 6 (Supplement) S115. Abstract Book centennial American Phytopathological Society's conference, July 26-31, 2008, Minneapolis, U.S.A.

### **Acknowledgement**

I hereby acknowledge the US Department of Agriculture, IBG, WSSA and SWSS for providing a travel grant to attend the 2009 joint meeting.

Table 1. Average Growth Rate (mm/day) of Isolates on Media Types

Media Types	Average growth rate (mm/day)
PCA	4.69 ± 0.13*
PDA	4.82 ± 0.21*
SA	4.40 ± 0.41*
TWA	13.13 ± 3.55
WHA	4.81 ± 0.50*
ZA	2.64 ± 0.12*

<sup>1</sup>Data represent average growth rate ± S.E.M of triplicate results derived as growth rate ( $\mu$ ) =  $\Delta S/\Delta T$ . Where  $\Delta S$  = Colony size (mm) and  $\Delta T$  = Time (Days). Medium with highest growth rate was compared to others using the student TTEST and ANOVA, where \* $P < 0.05$ .

Table 2. Neubaur count of *M. roridum* on media types.

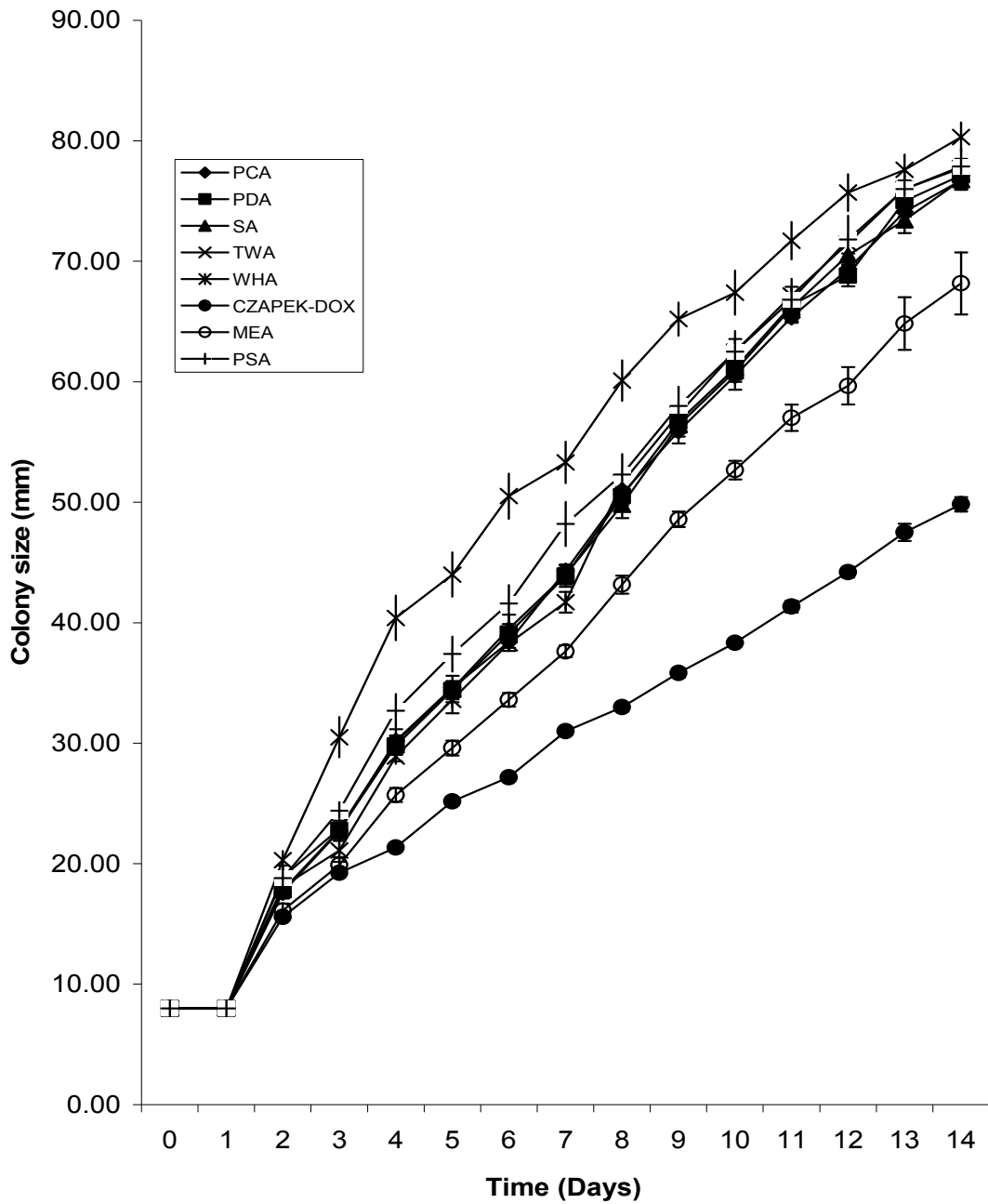
Media Type	Conidial yield (Spore /ml) x 10 <sup>7</sup>
ZA	0.160 ± 0.00
TWA	1.00 ± 0.00
WHA	3.90 ± 0.5
PCA	3.98 ± 0.01
PDA	5.37 ± 0.08
SA	26.4 ± 4.77
PSA	322.00 ± 8.52
MEA	2990.00 ± 7.89

Values are mean ± SEM of five replicate results.

Table 3. Neubaur count of *M. roridum* on nitrogen sources.

Nitrogen sources	Conidial yield (Spore /ml) x 10 <sup>6</sup>
Ammonium chloride	0.30 ± 0.00
Sodium nitrate (Czapek Dox)	1.85 ± 0.07
Ammonium nitrate	2.00 ± 0.01
Sodium glutamate	74.50 ± 0.58
Glutamine	91.10 ± 2.86

Values are mean ± SEM of five replicate results.



**Fig. 1. Growth Profile of *Myrothecium roridum* Tode (Culture IMI 394934) on Media Types. Values are mean  $\pm$  SEM of five replicate results.**



**Potential mycoherbicide fungi collected on arrowhead (*Sagittaria montevidensis*) in Brazil**

D. J. Soares, R. W. Barreto\*, B. V. Lima; Universidade Federal de Viçosa, Viçosa, MG, Brazil

*Sagittaria montevidensis* (arrowhead) is among the worst weeds of irrigated rice in Brazil, Australia and other parts of the world. The problem with infestations by this weed is worsening as infestations of irrigated rice by populations of this weed, which are resistant to ALS-inhibiting herbicides, are becoming widespread. Arrowhead is considered to be native from Brazil and work performed at UNESP Jaboticabal indicated that a fungus provisionally identified as *Cylindrocarpon* sp. might have potential as a mycoherbicide. Recently, a detailed survey of fungi attacking this aquatic weed in south and southeastern Brazil was completed and yielded nine fungal species, namely: the anamorphic hiphomycetes - *Alternaria alternata* (leaf spot), *Botrytis cinerea* (leaf blight), *Cercospora apii* (leaf spot), *Cercospora sagittariae* (leaf spot), *Colletotrichum gloeosporioides* (antracnose), *Plectosporium alismatis* (leaf spot) and a new species of *Pseudocercospora* (leaf spot); and two smut fungi, *Doassansiopsis deformans* and *Narasimhania alismatis*. All of them represent new host records or new geographic localities for occurrences of these fungi. Preliminary observations of the fungi in the field and in culture suggests that additionally to *Cylindrocarpon* sp. four species have potential for use as biocontrol agents against *S. montevidensis*, namely: *C. sagittariae*, *C. gloeosporioides*, *P. alismatis* and *Pseudocercospora*. All of these combine the conditions of being able to cause a severe disease in field conditions, growing well and sporulating abundantly in culture and belonging to groups of fungi that often show an adequate level of host-specificity or, in the case of *C. gloeosporioides*, include infra-specific taxa at the level of forma specialis that can be highly host-specific. Other fungi should be rejected at this stage of evaluation for the following reasons: *A. alternata* and *B. cinerea* - probably not host-specific and depending on injuries for infection; *C. apii* - known to be polyphagous and seemingly only weakly damaging to arrowhead; *D. deformans* and *N. alismatis* - found only occasionally and attacking few plants in each area and not showing significant impact on weed populations. Prospects appear very good for mycoherbicide development against arrowhead.

## **Status of *Phoma macrostoma*, a bioherbicide for broadleaved weed control in turfgrass**

Karen L. Bailey<sup>\*1</sup>, Stuart Falk<sup>2</sup>, and Steve. Lombardo<sup>2</sup>; <sup>1</sup>Agriculture & Agri-Food Canada, Saskatoon, SK, Canada, <sup>2</sup>The Scotts Company, Marysville, OH.

### **Introduction**

*Phoma macrostoma* is a fungus that was isolated from Canada thistle (*Cirsium arvense*) plants growing in several provinces in Canada. When applied to the soil, the fungus caused the Canada thistle plants to turn white and die. Host range testing showed that several dicotyledonous plants were susceptible but monocotyledonous plants were resistant. Dandelion (*Taraxacum officinale*) is another weed species that was highly susceptible to the fungus in preliminary greenhouse tests where emerging weed seedlings were killed more quickly than established plants. The objective of this study was to determine the least effective rate of the bioherbicide as a pre-emergent weed control for dandelion in turfgrass.

### **Materials and Methods**

*Phoma macrostoma* was grown using a solid state fermentation process and formulated as a granule for broadcast application as a pre-emergent bioherbicide. A mixture of grass seed (2.84 g/ m<sup>2</sup>) and dandelion seed (0.3 g/m<sup>2</sup>) were broadcast to 0.25 m<sup>2</sup> plots where the soil had been tilled, raked, and lightly packed. The bioherbicide was applied at sowing at rates ranging from 4X, 2X, 1X, 1/2X, 1/4X, 1/8X, and 0X. The experiments were conducted as a RCBD with 4 replications at five sites in Canada and one in the USA. Three trials were conducted for estimating dose response, six trials were conducted to obtain information comparing field formulations at various rates, and six other trials were used to evaluate crop tolerance to the bioherbicide. Data were collected on symptom expression (0-5 scale) and the percentage weed control using the Abbotts's transformation to express treated plots relative to the untreated control.

### **Results and discussion**

At rates greater than 1X, 100% weed control was attained in all trials and there was no harm to the grass. At the 1X rate, there was 80-100% weed control in 67% of the trials applying this rate, 60-80% control in 24% of the trials, and less than 60% control in 9% trials. At the 1/2X rate, there was 80-100% weed control in 29% of the trials, 60-80% control in 41% of the trials and less than 60% control in 30% of the trials. At the lower rates, there was less than 60% weed control in about 80% of the trials. The rate at which control was obtained over the season depended on the year and location. The maximum level of control was usually obtained by 28 days after application in 12 of 15 trials. In 3 trials, the maximum control was achieved by 42-56 days after applications. Control of emerging seedlings lasted throughout the season. For consistent pre-emergent weed control over multiple environments and years, the 1X application is recommended. The pre-emergent application would be suitable for preventing weed establishment and eliminating seeds germinating from the weed seed bank.

Table 1. The frequency of obtaining high (80-100%), medium (60-80 %) or low (<60%) dandelion control at various rates of *Phoma macrostoma* applied as a pre-emergent bioherbicide in 15 trials in 2007 and 2008.

Rate	Number of samples for 3 classes of weed control occurring in 15 trials (% of total sample)			Total Sample
	High control	Medium control	Low control	
4X	2 (100%)	0	0	2
2X	6 (100%)	0	0	6
1X	14 (66.7 %)	5 (23.8 %)	2 (9.5%)	21
1/2X	5 (29.4)	7 (41.2 %)	5 (29.4%)	17
1/4X	1 (9.1%)	2 (18.2%)	8 (72.7%)	11
1/8X	1 (6.7%)	1 (6.7%)	13 (86.6%)	15

## List of Delegates

### Ahrens, John

Connecticut Agric. Exp. Station  
PO Box 248  
Windsor, CT 06095  
Phone: 860-683-4985  
Fax: 860-683-4987  
Email: john.ahrens@po.state.ct.us

### Ashigh, Jamshid

3245 E. University Ave.  
Apt#1209  
Las Cruces, NM 88011  
United States  
Phone: (575) 646-2888  
Fax:  
Email: jashigh@nmsu.edu

### Bailey, Karen

Agriculture & Agri - Food Canada  
107 Science Place  
Saskatoon, SK S7N 0X2  
Canada  
Phone: (306) 956-7260  
Fax: (306) 956-7649  
Email: BAILEYK@AGR.GC.CA

### Barreto, Robert

Universidade Federal de Viçosa  
Dept de Fitopatologia  
Viçosa, MG 36571-000  
Brazil  
Phone: (31) 3899-2503  
Fax:  
Email: rbarreto@ufv.br

### Berner, Dana

Usda, Ars, Fdwsru  
1301 Ditto Ave.  
Ft. Detrick, MD 21702-5023  
United States  
Phone:  
Fax:  
Email: dana.berner@ars.usda.gov

### Biswas, Myrna

Andrews University  
Berrien Springs, MI 49104  
United States  
Phone: (269) 861-2782  
Fax:  
Email: myrnabiswas@gmail.com

### Bourdôt, Graeme

AgResearch Limited  
Lincoln Research Centre  
Cnr Springs Rd and Gerald St, Lincoln,  
Private Bag 4749  
Christchurch, Canterbury 8140  
New Zealand  
Phone:  
Fax:  
Email: graeme.bourdot@agresearch.co.nz

### Boyetchko, Susan

Agriculture and Agri-Food Canada  
Saskatoon Research Centre  
107 Science Place  
Saskatoon, SK S7N 0X2  
Canada  
Phone: (306) 956-7619  
Fax: (306) 956-7247  
Email: boyetchkos@agr.gc.ca

**Boyette, Doug**

Southern Weed Science Lab  
PO Box 350  
Stoneville, MS 38776  
United States  
Phone: (601) 686-2311  
Fax: (601) 686-9406  
Email: dboyette@ars.usda.gov

**Braverman, Michael**

IR-4 Project, Rutgers University  
500 College Road East  
Suite 201W  
Princeton, NJ 8536  
United States  
Phone: (732) 932-9575  
Fax: (732) 932-8481  
Email: braverman@aesop.rutgers.edu

**Bruckart, William**

USDA-ARS  
Foreign Disease-Weed Sci. Res  
1301 Ditto Ave.  
Ft. Detrick, MD 21702  
United States  
Phone: (301) 619-2846  
Fax: (301) 619-2880  
Email: william.bruckart@ars.usda.gov

**Charudattan, Raghavan**

University of Florida  
Plant Pathology Dept  
PO Box 110680  
Gainesville, FL 32611-0680  
United States  
Phone: (352) 392-3631  
Fax: (352) 392-5940  
Email: rcharu@ufl.edu

**Clemente, Thais Balbad**

Ouro Fino Group  
Rua: Prof Jose Loureiro JR, 139  
Ribeirao Preto/ Sao Paulo, Brazil  
Phone:  
Fax:  
Email: thais.clemente@ourofino.com

**Cutts, George**

149 Morton Ave.  
Apt. 15  
Athens, GA 30605  
United States  
Phone:  
Fax:  
Email: trey91@uga.edu

**Elzein, Abuelgasim**

University of Hohenheim (380)  
Institute of Plant Production & Agronomy  
Stuttgart, BW D-70593  
Germany  
Phone: 0049-711-45924346  
Fax: 0049-711-45922304  
Email: gasim@uni-hohenheim.de

**Englert, Clara**

North Carolina State University  
Kilgore Hall Box 7609  
Raleigh, NC 27695  
United States  
Phone:  
Fax:  
Email: caengler@ncsu.edu

**Evans, Harry**

CAB International, UK  
Bakeham Lane  
Egham, Surrey TW20 9TY  
United Kingdom  
Phone: +44 (0)1491-829149  
Fax: +44 (0)1491-82911213  
Email: h.evans@cabi.org

**Falk, Stuart**

The Scotts Company  
14310 Scottslawn Road  
Marysville, OH 43041  
United States  
Phone: (937) 644-7128  
Fax: (937) 644-7153  
Email: stuart.falk@Scotts.com

**Ghorbani, Reza**

Department of Agronomy  
Faculty of Agriculture  
Ferdows University of Mashhad  
Mashhad, Iran  
Phone:  
Fax:  
Email: ghorbani43@gmail.com

**Hashem, Abul**

Dept of Agriculture Western Australia  
Ctr for Cropping Systems  
Lot 12 York Road (P.O. Box 483)  
Northam, WA 6401  
Australia  
Phone: 618 9690 2000  
Fax: 618 9622 1902  
Email: ahashem@agric.wa.gov.au

**Heilman, Mark**

11550 N. Meridian St. Suite 600  
Carmel, IN 46032  
United States  
Phone: (252) 437-3282  
Fax: (317) 388-3336  
Email: markh@sepro.com

**Hynes, Russell**

Agriculture & Agri-Food Canada  
107 Science Place  
Saskatoon, SK S7N 0X2  
Canada  
Phone: (306) 956-7638  
Fax: (306) 956-7247  
Email: hynesr@agr.gc.ca

**Inman, Scott**

3280 Westerdoll Avenue  
Loveland, CO 80538  
United States  
Phone: (970) 663-0860  
Fax: (970) 669-1813  
Email: scin@novozymes.com

**Jayachandran, Krishnaswamy**

Florida International University  
Dept of Environmental Studies  
11200 SW 8th St  
Miami, FL 33199  
United States  
Phone: (305) 348-6553  
Fax: (305) 348-6137  
Email: jayachan@fiu.edu

**Johnson, Timothy**

14 Baldtop Heights  
Danville, PA 17821  
United States  
Phone: (570) 441-8775  
Fax:  
Email: tjohnson@marroneorganics.com

**Kang, Yuewei**

Novozymes  
5400 Corporate Circle  
Salem, VA 24153  
United States  
Phone: (540) 302-1224  
Fax:  
Email: yaok@novozymes.com

**Koo, Suk-Jin**

Moghu Research Center Ltd  
BVC #311, KRIBB  
Yuseong  
Daejeon, 305-333  
Korea, Republic of  
Phone: 011 824-2862-4951  
Fax: (011) 824-2862-4950  
Email: bioweed@naver.com

**León, Ramón**

EARTH University  
Apartado 4442-1000  
  
San Jose,  
Costa Rica  
Phone: (506) 713-0000  
Fax: (506) 713-0001  
Email: rleon@earth.ac.cr

**Lin, Jun**

Petro-Canada RCD.  
2489 N. Sheridan Way  
Mississauga, ON Canada  
Phone:  
Fax:  
Email: jliu@petro-canada.ca

**Lydon, John**

USDA/ARS/SASL  
Bldg. 001, Rm. 227  
Beltsville, MD 20705-2350  
United States  
Phone: (301) 504-5379  
Fax: (301) 504-6491  
Email: john.lydon@ars.usda.gov

**Mittal, Sunil**

Panjab University  
Centre for Environment  
Street No. 5, House No. 16734/B  
Bathinda, Punjab 151001  
India  
Phone: 9.198e+11  
Fax:  
Email: sunil.ceys@gmail.com

**Moran, Patrick**

USDA-ARS, BIRU  
2413 E Hwy 83  
Weslaco, TX 78596  
United States  
Phone: (956) 969-4846  
Fax: (956) 969-4888  
Email: pmoran@weslaco.ars.usda.gov

**Neal, Joe**

North Carolina State University  
Horticultural Science  
262 Kilgore Hall Campus Box 7609  
Raleigh, NC 27695-7609  
United States  
Phone: (919) 515-9379  
Fax: (919) 515-7747  
Email: joe\_neal@ncsu.edu

**Okunowo, Wahab**

Biochemistry Dept  
College of Medicine Univ of Lagos  
Idi Araba  
Surulere, 234-01  
Nigeria  
Phone:  
Fax:  
Email: modelprof@yahoo.com **Pandy,**

**Binod**

Florida International University  
Miama, FL 33199  
Phone:  
Fax:  
Email:

**Pitelli, Robinson**

EcoSafe  
Rua Monteiro Lobato, 856  
Jaboticabal SP, 14870-410  
Brazil  
Phone: 55-16-3203-3117  
Fax: 55-16-3202-2301  
Email: rapitelli@ecosafe.agr.br

**Porpiglia, Peter**

3238 Cutty Sark Way  
Melbourne, FL 32903  
United States  
Phone: (321) 409-7758  
Fax: (321) 409-7310  
Email: pporpiglia@tyratech.com

**Qiang, Sheng**

Nanjing Agricultural University  
Tongwei Road 6  
No. 1 Weigang  
Nanjing, 210095  
China  
Phone:  
Fax:  
Email: wvl@njau.edu.cn

**Rashed, Mohammad H.**

Department of Agronomy  
Faculty of Agriculture  
Ferdowsi University of Mashhad  
P.O.Box 91775-1163  
Mashhad Iran  
Phone:  
Fax:  
Email:

**Roskopf, Erin**

USDA/ARS  
2001 South Rock Road  
Ft. Pierce, FL 34945  
United States  
Phone: (772) 462-5887  
Fax: (772) 462-5986  
Email: eroskopf@ushrl.ars.usda.gov

**Russell, Andrew**

Herbicides & Plant Growth Regulators  
Section  
Health Canada  
Phone:  
Fax:  
Email: arussell@hc.sc.gc.ca

**Schmid, Tobias**

60 Woodglen Ct  
Oldsmar, FL 34677  
United States  
Phone:  
Fax:  
Email: schmid89@ufl.edu



**Shabana, Yasser**

1453 Fifield Hall  
Plant Pathology Dept.  
P.O. Box 110680  
Gainesville, FL 32611  
United States  
Phone: (352) 392-9055  
Fax: (352) 392-5940  
Email: yassershavana2@yahoo.com

**Shetty, Kateel**

Florida International University  
Depart. Environmental Studies  
11200 S W 8th St.  
Miami, FL 33199  
United States  
Phone: (305) 348-0178  
Fax: (305) 348-6137  
Email: shettyk@fiu.edu

**Shimizu, Aya i**

Lab for Sustainable Horticulture  
Miami Kyushu University  
Miyazaki 884-003. JPN  
Phone:  
Fax:  
Email: keny@nankyundai.ac.jp

**Smither-Kopperl, Margaret**

USDA-APHIS-PPQ  
835 South 192nd St. #1600  
Seatac, WA 98148  
United States  
Phone: (206) 878-6604  
Fax: (206) 870-8043  
Email: Margaret.L.Smithers-Kopperl@aphis.usda.gov

**Spesard, Bruce**

Bayer Environmental Science  
2 T W Alexander Dr  
Research Triangle Park, NC 27709  
United States  
Phone: (919) 549-2334  
Fax: (919) 549-3926  
Email: bruce.spesard@bayercropscience.com

**Sundhu-Kappal, Margaret**

USDA-APHIS-PPO  
Seal-otc WA 98'48  
Phone:  
Fax:  
Email: Margaret.L.sundhu-happal@aphis.usda.gov

**Vurro, Maurizio**

Institute of Sciences of Food Production.  
CNR  
Via Amendola 122/O  
Bari, I-70125  
Italy  
Phone: +39.080.5929331  
Fax: +39.080.5929376  
Email: maurizio.vurro@ispa.cnr.it

**Wahab, Okunowo**

Biochemistry Department  
College of Medicine  
University of Lagos  
Lagos State, Nigeria  
Phone:  
Fax:  
Email: modeprof@yahoo.com

**Walker, Lela**

North Carolina State University  
Campus Box 7609  
Kilgore Hall  
Raleigh, NC 27695  
United States  
Phone:  
Fax:  
Email: lcwalker@ncsu.edu

**Watson, Alan**

McGill University  
Plant Science  
21,111 Lakeshore Rd  
St Anne de Bellevue, QC H9X 3V9  
Canada  
Phone: (514) 398-7858  
Fax: (514) 398-7897  
Email: alan.watson@mcgill.ca

**Wu, Yun**

USDA Forest Service  
Forest Health Technology Enterprise Team  
180 Canfield St.  
Morgantown, WV 26505  
Phone:  
Fax:  
Email: ywu@fs.fed.us

**Xiang, Meimei**

Agriculture and Landscape College,  
Zhongkai  
University of Agriculture and Engineering,  
Zhongkai  
Road, Guanyzhou 510225, China  
Phone:  
Fax:  
Email: mm-xaing@163.com

**Xue, Guang**

Rm.a-409,weigangxi 18#  
Nanjing, Jiangsu 210007  
China  
Phone: 0086-25-84864864  
Fax: 0086-25-84864528  
Email: xueguang@jlonline.com

**Yamaguchi, Kenich**

Lab for Sustainable Horticulture  
Miami Kyushu University  
Miyazaki 884-003. JPN  
Email: kenyan@nankyundai.ac.jp

**Yamaguchi, Harumi**

Lab for Sustainable Horticulture  
Miami Kyushu University  
Miyazaki 884-003. JPN  
Phone:  
Fax:  
Email: kenyan@nankyundai.ac.jp

**Zdor, Robert**

Andrews University  
Price Hall, Dept. of Biology  
Berrien Springs, MI 49104  
United States  
Phone: (269) 471-6696  
Fax: (269) 471-6911  
Email: zdor@andrews.edu

**Zhang, Wenming**

Pest Management Regulatory Agency  
Health Canada  
Phone:  
Fax:  
Email: Wenming\_zhang@hc-sc.gc.ca

