

Title:

Buffelgrass Biocontrol in the Sonoran Desert with Foliar Pathogen Phytotoxic Metabolites

Principal Investigator:

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BCIP Contact:

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Amount Requested: \$43,000

Nonfederal Matching: \$43,300

Total Matching (including Federal): \$79,300

Project Justification/Urgency

Buffelgrass (*Pennisetum ciliare*, *Cenchrus ciliare*), a native of south and east Africa and southern Asia, is an important pasture grass in many semi-arid regions of the world including Texas and northern Mexico (Bogdan 1977, t'Mannetje and Kersten 1992). It has become highly invasive in some parts of its introduced range, particularly in the Sonoran Desert of southern Arizona (Bowers et al. 2006, Rutman and Dickson 2002, Stevens and Falk 2009), where it has infested thousands of acres of public and private lands, including Saguaro National Park and the Coronado and Tonto National Forests.

Increased fire frequency and intensity is a major risk associated with buffelgrass invasion, particularly into non-fire-adapted desert ecosystems such as the Sonoran Desert (Burquez-Montijo et al. 2002). Saguaro cactus and other stem succulents are particularly at risk from buffelgrass-associated wildfire, and there is a real danger of loss of these iconic cactus species if the spread and increase of buffelgrass in these ecosystems cannot be halted. This is one reason for the high level of public interest in buffelgrass control in places at the center of both cactus diversity and buffelgrass invasion, e.g., Tucson, Arizona. At present the only weapons available to deal with buffelgrass invasion into natural ecosystems are broad-spectrum herbicides (e.g., glyphosate) and physical removal with hand tools (Bean 2012).

Biocontrol has become an effective weapon to combat many weeds that invade natural systems. The biocontrol organisms used are most often insects, but fungal biocontrols have also been effective in some cases, for example, *Puccinea chondrillina* for biocontrol of *Chondrilla juncea* in Australia (Burdon et al. 1981). Buffelgrass is impacted by several fungal pathogens in its introduced range, but the direct use of these pathogens as biocontrol agents is problematic for at least two reasons. First, these pathogens cause leaf spot diseases (blasts or blights), which rarely reach epidemic levels that can severely damage buffelgrass plants because of their requirement for prolonged periods of high humidity. This constraint is even more severe in the Sonoran Desert, where buffelgrass has become invasive, than in areas of the Southwest where the species is extensively cultivated. Second, as buffelgrass is a commercially important forage grass in much of the Southwest, there is major societal resistance to the idea of developing a biocontrol that has the potential to damage buffelgrass pastures.

After several years of exploratory work, we have come to the conclusion that the best hope for control of buffelgrass in the Sonoran Desert lies in the development of a natural herbicide based on toxic metabolites produced by one or more of its fungal pathogens (Evidente et al. 2006). This herbicide would have a key advantage over currently available broad-spectrum chemical herbicides. The goal is to develop a herbicide with at least some degree of specificity, so that nontarget native species would be minimally impacted by treatments that effectively target and severely damage or kill buffelgrass.

We have identified three foliar pathogen species on buffelgrass in its North American range that are potential sources of candidate phytotoxins for buffelgrass control. The first of these is *Pyricularia grisea* (teleomorph *Magnaporthe grisea*). This organism is now considered to be distinct from the rice blast pathogen *M. oryzae*, but is known from a broad spectrum of warm season grass hosts. It is the only foliar pathogen that has been recorded to reach epidemic levels that severely damage buffelgrass in the field (Rodriguez et al. 1999, Perrott and Chakraborty 1999). An epidemic disease caused by this organism in Texas during a series of unusual weather years prompted a search for a blight-resistant buffelgrass strain (Diaz-Franco et al. 2007). This generalist pathogen exists in nature as a series of host-specific taxa and is known to include strains that produce host-specific phytotoxins, notably on crabgrass (*Digitaria sanguinalis*; Tsurushima et al. 2005). It therefore seems logical to investigate whether strains from buffelgrass also produce phytotoxins with some degree of selective action.

The second foliar pathogen under study is a *Cochliobolus* species closely related to *C. australiensis* and *C. hawaiiensis*. It is not closely related to *Cochliobolus* species that cause severe crop damage (Manamgoda et al. 2012). While this pathogen rarely impacts buffelgrass significantly in the field, we have determined that it produces highly toxic compounds that can severely damage buffelgrass, and that at least one of these compounds, named cochliotoxin (Fig. 1) shows some degree of selectivity (Masi et al. in review).

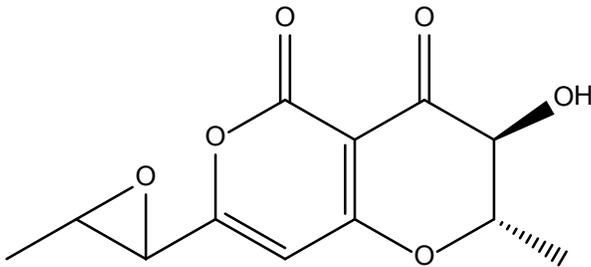


Figure 1. Cochliotoxin, a previously undescribed compound produced in liquid culture by a *Cochliobolus australiensis* strain isolated from diseased buffelgrass tissue. This phytotoxin showed higher toxicity in leaf puncture bioassays on buffelgrass than on two native nontarget grasses, (tanglehead: *Heteropogon contortus* and Arizona cottontop: *Digitaria californica*). (From Masi et al. in review).

A third foliar pathogen isolated from diseased buffelgrass collected at several locations in south Texas in 2014 is *Nigrospora cf. sphaerica*. This pathogen also has a broad host range that includes many warm season grasses and also some dicots. It is known to produce a wide array of secondary metabolites, but these have not been critically examined with regard to their toxicity to host vs. non host plants (Cutler et al. 1991, Kim et al. 2001, Metwaly et al. 2014).

Project Goal

Our primary goal is to determine whether foliar fungal pathogens on buffelgrass produce secondary metabolites that are more strongly phytotoxic on buffelgrass than on non-target hosts, and whether one or more of these phytotoxic metabolites has potential to be developed as a natural herbicide for buffelgrass control that would have reduced collateral damage on nontarget species.

Supporting Objectives for this Funding Cycle:

- 1) Determine whether one or more phytotoxins produced by *Cochliobolus cf. australiensis* from buffelgrass can significantly damage buffelgrass in whole plant bioassays, and whether they can damage buffelgrass more severely than non-target native grass species.
- 2) Expand testing of *Cochliobolus* secondary metabolites (and potentially phytotoxins from the other two foliar pathogens) to include more non-target species (e.g., shrubs, dicot herbs, other perennial grasses) from the Sonoran Desert, where buffelgrass invasion is most problematic.
- 3) Chemically characterize the secondary metabolites from two additional buffelgrass pathogens (*Pyricularia cf. grisea* and *Nigrospora cf. sphaerica*) and carry out seedling and leaf puncture bioassays on buffelgrass and non-target native species to determine whether any of these compounds have potential as additional candidate target-specific natural herbicides.
- 4) Carry out whole plant bioassays on buffelgrass with any promising phytotoxic metabolites produced by *Pyricularia* and *Nigrospora* to determine their potential for buffelgrass control.

Progress to Date

- We have explored the introduced range of buffelgrass in NA and acquired diseased tissue samples from multiple populations in Texas and Arizona in multiple years.
- We have obtained an extensive culture collection of the three principal pathogens found on buffelgrass in the introduced range.
- We have screened the culture collection for the production of secondary metabolites in liquid culture in terms of ability to impact buffelgrass seed germination and seedling growth.
- We have carried out studies to optimize liquid cultural conditions for the three pathogens.
- We have produced sufficient quantities of the filtrate lyophilates of the most promising strains to determine that the organic extracts of lyophilates of all three buffelgrass pathogens are extremely toxic to buffelgrass seeds and seedlings.
- For one of these pathogens, *Cochliobolus cf. australiensis*, we have carried out full chemical characterization of the lyophilate organic extract and have identified several strongly phytotoxic compounds, one of which (cochliotoxin) is previously undescribed.
- We have also produced solid wheat cultures of *Cochliobolus* and have determined that the fungus produces the same suite of secondary metabolites in this culture system as in liquid culture. This could be a method for producing key phytotoxins in larger amounts.
- We have completed seedling and leaf puncture bioassays of these compounds from *Cochliobolus* on buffelgrass and two nontarget host grasses and have shown that at least cochliotoxin has more negative impact on buffelgrass than on two native grasses in leaf puncture bioassays.
- We have a paper submitted to the peer-reviewed journal *Journal of Natural Products* describing this research and its results (Masi et al. in review).

Research in Progress

- We have on hand a larger production of lyophilate of the selected *Cochliobolus* strain. This will be used to obtain sufficient quantities of cochliotoxin and other phytotoxic compounds produced by this fungus to enable screening on additional nontarget hosts and in whole plant bioassays.
- We have produced sufficient lyophilate of selected strains of the other two pathogens (*Pyricularia cf. grisea* and *Nigrospora cf. sphaerica*) to begin chemical characterization and subsequent pure compound bioassay studies.
- We also have the opportunity to include multiple strains of these three pathogens in a genome sequencing project that will give us a much more definitive idea of how they are related to known strains on other

host species, and also to examine secondary metabolite gene clusters that can give clues to phytotoxin production. These strains can be included at minimal cost (<\$2000) in an Illumina sequencing run that primarily involves fungal species of interest for another project. We already have funds on hand to initiate and complete this genomics work.

Research Approach

We will continue with the research approach described above, with preliminary screening of liquid culture filtrates of strains in our collection, production of lyophilate for extraction with different organic solvents, and bioassays to determine the toxicity of the extract. We will also produce key strains of each pathogen in solid wheat seed culture as mentioned earlier for *Cochliobolus*. It is known that the a fungal strain grown on solid medium is often able to produce bioactive metabolites different from those produced in liquid culture, for example, the seed pathogen *Pyrenophora semeniperda* (Masi et al. 2014 a, b, c, d).

We will then perform bio-guided chromatographic purification of the phytotoxic compounds, which will then be characterized using chemical and spectroscopic methods. In bio-guided purification, filtrates, extracts, chromatographic fractions, and pure compounds are checked for toxic activity using bioassay at each step of the purification process. This is to ensure that the focus is on identification of secondary metabolites that are phytotoxic, as many fungi also secrete nontoxic substances in culture. For already known compounds, preliminary investigation (ESI-MS, ¹H-NMR and OR) is sufficient for chemical characterization. For new phytotoxins, more extensive NMR work will be needed, including the use of 1D and 2D ¹H and ¹³C NMR spectra and in particular COSY, HSQC, HMBC and NOESY spectra. High-resolution ESI-MS will be performed to obtain the molecular formulas and molecular weights. X-ray diffractometric analysis could be used to determine structure and the relative configuration if suitable crystalline forms of the pure compound are obtained.

Pure compounds will then be bioassayed in seedling and leaf puncture tests. Once we know that we have a compound that is highly phytotoxic on buffelgrass, we will produce more of this compound in culture for whole plant bioassay. We will also examine the specificity of each compound in terms of the magnitude of its effect on buffelgrass relative to non-target native species.

The hemisynthesis of some key derivatives will be performed to confirm the structure of the most promising phytotoxins and to also carry out structure-activity relationship studies aimed to finding derivatives that have increased activity, stability and specificity. Such a compound could potentially be used in formulations with high solubility in water which is important for practical application.

Expected Products and Outcomes

By the end of this phase of the project, we will know what phytotoxic compounds are produced by the three principal foliar pathogens on buffelgrass in the North American range. We will know how effectively these compounds can damage buffelgrass in seedling, leaf puncture and whole plant bioassays. We will also know whether any of these compounds exhibit some degree of specificity in terms of their ability to damage buffelgrass relative to non-target native species.

Future Research

If we are successful in identifying one or more fungal phytotoxins with the ability to significantly and selectively damage buffelgrass, the next steps will be:

- Work on the bioherbicide delivery system—this could include researching chemical modifications to the molecule to render it more soluble in water without affecting its herbicidal activity and examining its stability in different kinds of storage. A suitable derivative that shows increased activity and stability could be prepared in a delivery system using advanced methods based on nanoparticles and cyclodextrin encapsulation (Duchene et al. 1999, Vyas et al. 2008).
- Develop the technology to produce the phytotoxin in large quantities, either through scaled-up liquid or

solid culture technology or possibly through chemical synthesis.

- Team with a commercial entity specializing in the production of similar products to develop a commercially viable product for buffelgrass control.

Literature Cited

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PI Responsibilities:

Dr. Masi is currently under contract with the Biotechnology and Biological Control Agency (BCBA), but carries out his research activities (purification and chemical characterization) in the laboratory of Prof. Evidente at the University of Naples (UNINA). Culture production and seedling bioassays are carried out under the direction of Dr. Meyer at the USFS Shrub Sciences Laboratory (SSL), while leaf puncture and whole plant bioassays are carried out at under the direction of Dr. Cristofaro and Dr. Masi (BBCA). We plan to continue these arrangements. Dr. Masi's salary is currently funded through April 30, 2017. The requested funding cycle will cover salary for Dr. Masi through February 28, 2018. In lieu of other new funding, his activities during the last seven months of the grant will be confined to data analysis, manuscript preparation, and coordination with possible industry partners.

Time Period	UNINA	BBCA	SSL
4/1 - 6/30/17	PG LC phytotoxin purification/ characterization	LBA BG for PG purification WPBA CA (BG, AC, TH)	SBA BG for PG purification Obtain seeds/protocols for full native nontarge panel Produce SC of PG, NS
7/1 - 9/30/17	NS LC phytotoxin purification/ characterization CA structure activity studies MS prep	LBA BG for NS purification LBA BG for CA structure activity studies LBA CA full nontarget panel WPBA PG (BG, AC, TH)	SBA BG for NS purification SBA BG for CA structure ac- tivity studies Produce cultures as needed MS prep
10/1 - 12/31/17	PG SC phytotoxin purification/ characterization PG structure activity studies MS prep	LBA BG for PG purifica- tion LBA BG for PG structure activity studies LBA PG full nontarget panel	SBA BG for PG purification SBA BG for PG structure activ- ity studies Produce cultures as needed MS prep
1/1 - 3/31/18	NS SC phytotoxin purification/ characterization NS structure activity studies	LBA BG for NS purification LBA BG for NS structure activity studies	SBA BGfor NS purification SBA BG for NS structure activ- ity studies
4/1 - 6/30/18	MS prep	WPBA NS (BG, AC, TH) LBA for NS full nontarget panel	MS prep
7/1 - 9/30/18	MS prep/industry coord.	MS prep/industry coord.	MS prep/industry coord.

LBA=leaf puncture bioassay; SBA=seedling bioassay; WPBA=whole plant bioassay
LC=liquid culture; SC=solid culture; BG=buffelgrass; AC=Arizona cottontop; TH=tanglehead
CA=*Cochliobolus australiensis*; PG=*Pyricularia grisea*; NS=*Nigrospora sphaerica*

Budget/Cost-sharing

Funds Requested:	
Masi (PI) Salary(w/benefits) 10 months @ \$3500/month	\$35,000
Supplies	\$6,000
Travel (Masi to US for coordination meetings)	\$2,000
Total Requested	\$43,000
Matching Funds:	
USFS Rocky Mountain Research Station Shrub Sciences Lab (Federal)	
Meyer (PI) Contributed salary (w/benefits) 6 wks @\$3,500/wk	\$21,000
Clement (Technician) Contributed salary (w/benefits) 12 wks @\$1,250/wk	\$15,000
Biotechnology and Biological Control Agency (Nonfederal)	
Waived overhead @10% of net	\$4,300
Contributed technical assistance (MS student, U Rome)	\$15,000
University of Naples Federico II (Nonfederal)	
Contributed technical assistance (MS student UNINA)	\$15,000
Evidente (PI) salary (w/benefits) 2 wks @\$3,000/wk	\$6,000
Contributed supplies	\$3,000
Nonfederal Matching	\$43,300
Total Matching	\$79,300
Total Funding Requested and Matching	\$122,300

Note: Funding from BCIP is requested only in direct support of the chemistry research to be carried out by Dr. Masi. The Shrub Sciences Lab will contribute PI and technician permanent salary to carry out the culture and bioassay work and to take part in planning and manuscript preparation. Sufficient funding from an earlier grant is in hand to cover the cost of supplies. BBCA will contribute waived overhead and also technical assistance in the form of a master's student to carry out the bioassay work. UNINA will contribute PI salary for Prof. Evidente and technical assistance in the form of a master's student to assist Dr. Masi.