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Identification of Fungal Species that Trigger Invasive Plants

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Table of Contents

ABSTRACT

Objectives

The invasive plant species *Imperata cylindrica* (cogongrass) is at the top of the U.S. Department of Agriculture's priority list for research, as it is projected to cover more land mass in the southeastern United States than the famous kudzu in the next 5-10 years. The goal of this research is to find potential growth inhibiting relationships between pathogenic fungal species and the cogongrass plant. Cogongrass, Japanese bloodgrass (an ornamental, less invasive version of cogongrass), and revertant plants were all analyzed for growth effects due to fungal pathogens.

Methods

To perform this research, fungal samples were collected from the three types of plants. DNA from these sample was extracted, purified, and sequenced. These sequences were then compared to databases to determine the species of fungus.

Results

There were top three fungal species represented in the resulting data—*Fusarium proliferatum*, *Fusarium oxysporum*, and *Fusarium fujikuori*. These three were shown to be present in most of the plants tested; this includes plants from all three types listed above. The only exception was in several of the revertant plants, in which there were found much higher occurrences of *Fusarium oxysporum*.

Conclusions

Although there appears to be some pattern of higher occurrence in revertants, the results are not significant enough to hail *Fusarium oxysporum* as a cause of reversion. Also, there were no fungal species that occurred particularly higher in Japanese bloodgrass, which would indicate a pathogenic relationship that causes growth inhibition. Therefore, the next steps for research will be to further analyze the three fungal species mentioned above to better understand what roles they might play in the metabolism of *Imperata cylindrica*. In addition, plant samples of the three types of *Imperata cylindrica* should be taken from various locations of southeastern United States and other locations worldwide to compare.

LIST OF ILLUSTRATIONS

RESEARCH PAPER

Introduction

Imperata cylindrica, more commonly known in the United States as cogongrass, is a Japanese plant with a history of aggressive growth. This species was brought over to the United States and has thrived in the warmer climates of southeastern states, such as Alabama, Mississippi, Georgia, South Carolina, etc. So much so, in fact, that it has found a long-standing place on the United States Department of Agriculture's most noxious weeds list, as well as gained top priority on their commissioned research endeavors. This is due to the potential of cogongrass to completely take over large amounts of land mass, choking out all other plants species (both invasive and non-invasive) and even local fauna. (Loewenstein and Miller 2007) In turn, this aggression can lead to drastic changes in ecosystems. One major impact of changes in the biological landscape is a much larger fire hazard, due to the longer tails of grass grown at much higher densities (see Figure 1-1). (Setterfield et al. 2013) In addition, with global warming and increased CO_2 in the atmosphere, environmental conditions are only increasing the chances of these plants thriving currently. (Runion et al. 2016)

Figure 2-1: Cogongrass is a fire hazard due to its long-tailed and growth density properties (Loewenstein and Miller 2007)

However, not all species of *Imperata cylindrica* are this invasive. Japanese bloodgrass, for instance, is a type of *Imperata cylindrica* that is often used in landscaping for its ornamental properties. The difference between Japanese bloodgrass and wild-type cogongrass is not limited to the outward physical characteristics of the plants but extends to metabolic growth mechanisms that are somehow slowed or inhibited in the non-invasive Japanese bloodgrass. In this case, variegation and presence of certain pigments have been pointed to as modes of slowed growth (Anderson et al. 2006), while others look to pathogenic relationships with fungi as a potential mechanism (Xuan et al. 2009). However, these types of research have not offered applicable solution to the problem, as many Japanese bloodgrass plants have been known to revert to the wild-type aggressive cogongrass form. Subsequent studies have been performed to sequence the genomes of all three types of *Imperata cylindrica*—wild-type cogongrass, Japanese bloodgrass, and revertant—to determine if there is any significant difference. (Cseke and Talley 2012)

Much research has been done regarding metabolic pathways of increased growth patterns, environmental affects, and growth suppression of cogongrass to achieve non-invasive characteristics like Japanese bloodgrass or to kill the plant altogether in undesired areas. In the rhizomes of cogongrass, several acidic growth inhibitors were discovered; these serve the purpose of eliminating surrounding plants to achieve greater nutrition and greater invasiveness. (Xuan et al. 2015) However, these are not easily targeted as they function as a bioproduct of the plant's normal metabolism. In addition, imazapyr herbicides and other similar bio-herbicides were tested against cogongrass growth rate, but none showed significant long-term effects, except in expensive combinations of many chemicals. (Holzmueller and Jose 2010) (Aulakh et al. 2014) In fact, studies have even been conducted to find a useful application of the plant for commercial profit. And while it was determined that cogongrass does produce a commercially useful amorphous bio-silica compounds, the results did not indicate that the quantity produced was significantly high enough to lessen the war against its invasive properties. (Kow et al. 2014)

However, there has been some indication in other invasive plant species that pathogenic fungi play a large role in slowing growth/metabolic properties without necessarily destroying the plant itself. (Castro de Souza et al. 2016) To this effect, the following research was conducted. The goal was to analyze relationships between *Imperata cylindrica* and various fungal species and to determine identities of possible candidates for a species hindering growth. It was hypothesized that there would be one or more fungal species proliferating in the Japanese bloodgrass (non-invasive) plants that hindered the plants from reaching their full invasive potential. And, due to previously known relationships, it was suspected that, in general, the species would come from the genus *Fusarium/Gibberella* and specifically be identified as *Fusarium fujikuori*.

Materials and Methods

This section describes in detail the steps taken to perform the experiment in question (growth, extraction, and analysis of fungus from Japanese bloodgrass, revertant, and cogongrass runners) and methods used to analyze the resulting data.

Preparation for Runner Collection

Three types of plant cultivation media—Murashige and Skoog (MS), potato dextrose agar (PDA), and woody plant medium (WPM)—were plated, containing plant vitamins. Three solutions for each type of plant runner were prepared—soap solution with 0.1% tween20 detergent, 1% sucrose solution, and 20% bleach solution.

Repotting of Plants and Collection of Runners

Cogongrass, revertant, and Japanese bloodgrass plants (2 of each type—1 from South Carolina and 1 from Missouri) were removed from their pots; their roots were separated, and

6-inch cutting were taken from the plants (not including the root tip) and placed into conical tubes. These cuttings were washed in the soapy solution mentioned above and rinsed 5 times in tap water. The plants were re-potted.

Surface Sterilization of Runners

Two of the tubes were then stored at 4°C. The remaining four tubes were soaked in the 1% sucrose solution for 24 hours after which, using sterile methods*, they were rinsed with 70% ethanol and covered with the 20% bleach solution for 30 minutes. The cuttings were washed with 3 times with sterile ddH_2O. Two more tubes of cuttings were stored at 4°C for future use.

Preparation of Vascular Tissue Plug

Using sterile methods*, the ends of each cutting were removed on a sterile surface and discarded. The remaining portion of each cutting was separated into sections each of approximately length 2-3 mm. With the cut end facing down, a 1mm disk of inner vascular tissue was removed from the center of the cuttings. Five to seven plugs from each type of plant were plated onto the WPM, PDA, and MS media, which were then carefully labeled. (3 plants x 3 replicas x 3 plates x 2 locations = 54 plates)

The plants were firmly wrapped in parafilm and stored at room temperature (approximately 37°C) in the dark. An additional 3 plates of media were included as controls. The plates were kept under this condition and checked daily until adequate bacterial or fungal growth was evident. At this point, the plates were transferred to a refrigerator and eventually freezer to slow their growth.

Propagation of Fungal and Bacterial Species

Using sterile methods*, each bacterial or fungal colony grown on the plant media from the runners was transferred to a new plate of the same medium type. All plates were carefully labeled and stored under the same conditions (sealed in parafilm, kept under room temperature in the dark). As before, the plates were removed from these conditions when abundant growth was evident and then stored in the refrigerator.

Sample Collection

Using sterile methods*, the plates were observed for possible fungal growth (especially *Fusarium/Gibberella fujikuori*). For those containing these kinds of growth, 2-3 small squares of the colony were removed via scalpel and placed in labeled micro-centrifuge tubes. All sample plates were resealed in parafilm and returned to the refrigerator. The colony sample tubes were stored in the freezer. Table 2-1 gives details on the samples collected.

Table 2-7: Details on collected samples. Sample ID indicates state collected, type of plant, and plant sample, respectively.

Sample #	Sample ID	Media
1	MO-JBG-2	MS
2	MO-CG-2	PDA
3	MO-CG-2	WPM
4	MO-CG-3	MS
5	MO-CG-1	MS
6	MO-REV-3	MS
7	MO-REV-1	PDA
8	MO-REV-1	MS
9	SC-CG-2	PDA
10	SC-CG-3	MS
11	SC-CG-1	WPM
12	SC-CG-1	PDA
13	SC-CG-3	PDA
14	SC-CG-3	WPM
15	SC-CG-2	WPM

DNA Extraction

A CTAB (cetyltrimethyl ammonium bromide) extraction protocol was used to extract DNA from the fungal samples. A 2% CTAB extraction buffer was prepared at pH 8, combined with 2% PVP-40 (polyvinylpyrrolidone with molecular weight 40) solution, and stored at room temperature. Frozen fungal samples (2-3 squares taken from colonies) were separately ground in liquid nitrogen. The resulting powder was placed into screw cap tubes. After mixing the powder with 500 µL of the CTAB buffere/PVP-40 solution and 2.5µL of β-mercaptoethanol in a hood, the tubes were incubated at 65°C for 30 minutes, shaking the sample every 10 minutes. To this solution, 500 µL of pheno-isoamyl-chloroform was added. The tubes were agitated on an electric rocker for 20 minutes.

Using a centrifuge, the tubes were spun down at maximum speed for 5 minutes. The top layers of supernatant were removed and combined with 15 µL of 5M potassium acetate and 500 µL of ice cold 2-propanol. Any extra layers of supernatant were removed and prepared with the top layer. The new supernatant tubes were centrifuged at maximum speed for 3 minutes. The supernatant was removed from each, and the pellets were combined with 500 µL of ice cold 70% ethanol, after which the tubes were incubated for 5 minutes at room temperature (approximately 37°C). These solutions were centrifuged at maximum speed for 1 minute. Again, the supernatant was removed from each tube, the tubes were centrifuged at maximum speed for 1 minute, and the final pellets were isolated. After drying completely at approximately 65°C, the pellets were reconstituted with 50 µl of tris-EDTA buffer. All sample pellet solutions and supernatants were labeled and placed in the freezer for storage.

Samples were thawed for Nanodrop testing. To prepare, samples were combined with 50 µL increments of tris-EDTA buffer and heated at 65°C as needed to thaw and dissolve properly. Using a Nanodrop spectrophotometer, the samples absorbance values (ng/µL), their 260/280 readings, and their 260/230 readings were measured and recorded. Between each reading, the spectrophotometer was cleaned lens paper, and the spectrophotometer was blanked between each set. The samples that measured high concentrations were diluted until they reached optimal values.

Polymerase Chain Reaction (PCR)

Under sterile conditions*, the sample solutions were prepared for PCR using a F7/R7 primer set and 2 µL of each sample in PCR tubes. A negative control and a positive control were also prepared for comparison. After mixing all PCR tubes, 35 cycles of PCR were run (see Table 2-2). The PCR products were then tested by running them on an agar gel at 100 V with a sodium borate buffer. The gel was placed on a transluminator and exposed to UV to check for DNA.

Table 8-2: Detail of action in polymerase chain reaction sequence

	Temperature (°C)	Time (sec)	# of Cycles
HotStart	95	120	1
Denature	95	30	34
Anneal	51	30	34
Extension	68	90	34
Final Extension	68	300	34
Hold	4	As Needed	N/A

Preparation for Sequencing

The PCR products were transferred to new tubes and shaken with 36 µL magnetic beads. Twice, the mixture was combined with 200 µL of 70% EtOH and isolated from the supernatant. After drying, 40 µL of tris-EDTA buffer was added to the tubes, and the resulting supernatant was removed. Nanodrop analysis was performed on the supernatant. Samples were concentrated at approximately 5 ng/µL using the PCR product and tris-EDTA buffer centrifuged at maximum speed for 10 seconds. Portions of these solutions were combined with forward and reverse primers for every sample collected. These solutions were sent to Eurofins sequencing company.

Analysis

All resulting forward and reverse sequences from Eurofins were compared with the NCBI BLAST database to test for known fungal identities. Hits were identified and categorized according to the type and location of the plant from which they originated. These results were recorded in tables and described below in the Results section.

*Sterile Methods

The sterile methods mentioned above consisted of using a sterile hood, swiping all surfaces before and after use with 70% EtOH, flaming appropriate tools and materials, using sterile gloves, etc. The purpose was to minimize or eliminate contamination of the DNA samples.

Results

The following text, tables, and figures detail and explain the resulting data from the research described above.

Wild-type Cogongrass

In the wild-type cogongrass, there were three main species that showed the greatest amount of hits—*Fusarium proliferatum*, *Fusarium oxysporum*, and *Fusarium fujikuori*. In that order, the data generally showed the number of hits for similarity in DNA sequences. (*Fusarium proliferatum* typically had the greatest number of hits in similarity.) The only exception to this was one of the cogongrass plants from South Carolina. In this plant, the overwhelming majority of hits identified as *Fusarium oxysporum*. It is important to not, as well, that these statements were mostly consistent between the forward sequence analysis and the reverse sequence analysis for every sample. This data is demonstrated in Table 3-1.

Table 9-1: Sequence hits on fungi extraced from wild-type cogongrass

	Average Percentage of Hits		
Sample #	*F. proliferatum*	*F. oxysporum*	*F. fujikuori*
2	34%	23%	10%
3	37%	21%	13%
4	24%	25%	10%
5	34%	23%	10%
9	35%	23%	12%
10	40%	24%	11%
11	38%	23%	11%
12	37%	23%	10%
13	38%	18%	10%
14	0%	91%	0%
15	39%	23%	13%

Japanese Bloodgrass

For the Japanese bloodgrass plants, there were much fewer plants that clearly showed any type of relationship with any fungal species. Due to this, only one viable fungus sample was available for DNA extraction and sequencing. Interestingly enough, it showed almost the exact same numbers of hits for *Fusarium proliferatum*, *Fusarium oxysporum*, and *Fusarium fujikuori* as was shown in the

pattern for wild-type cogongrass. In this instance, the forward sequencing analysis and reverse sequencing analysis both agreed on the top three fungal species identified and showed similar number of hits. The data for this is compiled in Table 3-2.

Table 10-2: Sequence hits on fungi extraced from Japanese bloodgrass

	Average Percentage of Hits		
Sample #	*F. proliferatum*	*F. oxysporum*	*F. fujikuori*
1	33%	23%	12%

Revertants

The remaining sample were collected from revertant plants (ones that were a non-invasive form, such as Japanese bloodgrass, and through an unknown mechanism reverted to the highly invasive wild-type cogongrass). Just as in the Japanese bloodgrass category, there were barely any representation of fungi propagated from the plant roots. In fact, only three of the revertant plants led to the production of a pathogenic fungal relationship. However, the results are somewhat different than in the other two types of *Imperata cylindrica*. Two out of the three DNA samples from fungi of revertant plants showed an overwhelming number of hits for *Fusarium oxysporum*, while the remaining fungus sample followed after the pattern of the species associated wild-type cogongrass and Japanese bloodgrass. This final revertant sample contained hits for all three of the top species matches—*Fusarium proliferatum*, *Fusarium oxysporum*, and *Fusarium fujikuori.* These results can be seen in Table 3-3.

Table 11-3: Sequence hits on fungi extraced from revertant cogongrass plants

	Average Percentage of Hits		
Sample #	*F. proliferatum*	*F. oxysporum*	*F. fujikuori*
6	0%	85%	0%
7	39%	21%	13%
8	0%	81%	0%

In addition, the analyzed data did not show any significant difference of sequencing results based on whether the original plants came from South Carolina or from Missouri. Approximately half of the overall samples came from South Carolina and the other half from Missouri; two of the three samples with overwhelming numbers of *Fusarium oxysporum* hits originated in Missouri and the remaining sample originated in South Carolina.

Remaining Samples

Sample 16 and 17 were included as positive and negative control for the PCR and subsequent sequencing. There results showed overwhelming amounts of *Fusarium oxysporum*, as indicated in Table 3-4.

Table 12-4: Sequence hits on positive and negative controls, respectively

	Average Percentage of Hits		
Sample #	*F. proliferatum*	*F. oxysporum*	*F. fujikuori*
16	0%	87%	0%
17	0%	94%	0%

Conclusion

As the data shows, there is definitely clear evidence to show that there are three particular fungi species that *Imperata cylindrica* consistently has pathogenic relationships with, regardless of the location in which the plant grows. This is true of all three types of *Imperata cylindrica* studied—wild-type cogongrass, Japanese bloodgrass, and revertant plants. The three species that showed an overwhelming number of sequence hits during analysis of the data are *Fusarium proliferatum*, *Fusarium oxysporum*, and *Fusarium fujikuori.* As shown in Tables 3-1, 3-2, and 3-3, all of these species would be good candidates for future research in general.

However, the one species that stood out above the rest in uniqueness was *Fusarium oxysporum*. Instead of it proliferating mostly in the non-invasive Japanese bloodgrass plants, which would implicate it as a potential inhibitor of aggressive growth, there were actually instances overwhelming *Fusarium oxysporum* sequence hits found in both wild-type cogongrass and revertant plants, although the occurrence of this was much higher in the revertant. This is still insufficient evident though to officially conclude that *Fusarium oxysporum* is for sure a cause of any particular metabolic change in any of the types of *Imperata cylindrica*. However, it does warrant future research and investigation into its widespread occurrence and potential effects on cogongrass.

In conclusion, it is important to note that while data was obtained in this research, the analysis of said data indicates one or more potential incidents of contaminations. This can be seen in the positive and negative controls; the negative control should not have any hits of similar DNA sequences, as it should be free of DNA. In addition, the haphazard occurrences of *Fusarium oxysporum* with little pattern as to relationships with a specific type of cogongrass indicates that this species is the contaminant. This could have happened via improper sterilization techniques, prior contamination of chemicals used, improper storage of chemicals or samples, misuse of pipettes/pipetting techniques. The resulting contamination, if proven to be such, would necessitate repetition of procedures on the same or similar plants for more accurate and applicable sequencing data and analysis.

Although this probable contamination seems apparent, the results are still not totally negated. As mentioned above, the analysis still gives reason to assume that the three *Fusarium* species identified could have major impacts on the plant—perhaps increasing its ability to growth, instead of inhibiting. Either way, the hypothesis is not necessarily proven or

disproven, and future research will be required to give definitive answers on any mechanisms regarding growth patterns in *Imperata cylindrica.* It is the hope of the researcher that the work done here can serve as a foundation for future cogongrass studies and, eventually, lead to victory over the invasive species in the southeastern United States.

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SUPPLEMENTARY DATA