THE UNIVERSITY of TULSA

Office of Research and Sponsored Programs

August 31, 2018

Bob Morrissey, Executive Director
National Watermelon Association
5129 South Lakeland Drive, Suite 1
Lakeland, Florida 33813

Dear Mr. Morrissey:

Please find attached a project proposal entitled "Suppression of Fusarium Wilt of Watermelon by Mycoviruses" to be considered for funding under the RFP for 2019.

Dr. Akhtar Ali, Associate Professor of Biology at The University of Tulsa, is a plant virologist having more than 25 years of experience working with the molecular characterization of various viruses in cucurbits particularly watermelon and other vegetables. His background is international in scope and includes a PhD thesis, "Pathology and molecular characterization of pea seed-borne mosaic virus", at the University of Adelaide, Australia. He received exceptional experience through three Postdoctoral Research appointments before coming to the University of Tulsa in 2007 (i.e., University of Adelaide, Australia; Tohoku Agricultural Research Centre, Morioka, Japan; and the nationally renowned Samuel Roberts Noble Foundation, Ardmore, Oklahoma.).

Recently, Dr. Ali was awarded a US Fulbright fellowship to Japan where he worked on the characterization of mycoviruses infecting Fusarium species at the University of Okayama, Japan. The proposed project will directly benefit from this scientific collaboration. This new proposal intends to better protect watermelon crops through specific objectives outlined in the proposal regarding mycoviruses of Fusarium species that cause Fusarium wilt of watermelon. The team of four researchers who wish to be a part of this collaboration without asking for monetary support, bring together amazing experience and a wealth of resources to Dr. Ali and the watermelon community overall.

I believe you will find everything in order per RFP guidelines, and we can assure you that this proposal has received all appropriate approvals within the University system. If you have any questions of a technical nature, you are welcome to contact Dr. Ali directly (918.631.2018 or akhtar-ali@utulsa.edu). Administrative or contractual types of questions should be directed to Ms. Adrienne Blalack, Director of Pre-award Services, Office of Research and Sponsored Programs at The University of Tulsa. Ms. Blalack can be reached at 918.631.2480 or adrienne-blalack@utulsa.edu.

On behalf of the University of Tulsa, thank you for your consideration.

Linda Golden
Sr. Coordinator of Pre-award Services

cc: Debbie Newton, Director of Research and Sponsored Programs
and Authorized Representative

Project Proposal to the National Watermelon Association

Suppression of Fusarium Wilt of Watermelon by Mycoviruses

August 2018

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Other Research Collaborators

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Dr. Charlie Biles
Professor of Biology
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Dr. Benny D. Bruton (retired)
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1. Cover letter (attached)

2. Details of the project

A. Background and significance

Cucurbits, particularly watermelon, are cash crops that can be grown in any state in the United States. Despite this, more than 70% of watermelon production is concentrated in the southern United States (U.S.) especially Florida and Texas, the leading states in term of total watermelon acreage and production.

Watermelon is subject to a variety of diseases caused by plant pathogens, particularly fungi and viruses, and have a significant impact on the yield by reducing yield or quality of product, which subsequently causes millions of dollars in losses to the watermelon industry. Among those diseases, Fusarium wilt is a widespread disease in watermelon throughout the United States (Keinath and Hassell, 2014). It is one of the most economically detrimental diseases affecting watermelon because it limits triploid (seedless) watermelon production (Zhou et al., 2010) and can result in 100% yield loss.

Fusarium wilt is caused by *Fusarium oxysporum* f. sp. *niveum* (FON) which is a soil borne fungus that can survive in the soil for 15-20 years in the absence of watermelon plants. FON has four known races (0, 1, 2 and 3) which can be distinguished by their pathogenicity on watermelon cultivars. Although many watermelon cultivars have some resistance to FON, the emergence of new races of FON have reduced the efficiency of the resistance genes in watermelon cultivars.

Various management strategies against FON has been applied, including resistant varieties, soil fumigation with methyl bromide, cultural practices and crop rotation. However, all the existing methods have various disadvantages and are sometimes effective but not always. None of the management methods is long lasting in order to protect watermelon from the effects of FON. For example, the new races of FON can break the resistance, there is a ban on the fumigation of methyl bromide in the US, and the expenses of fungicides are high and it results in environmental pollution. In order to protect watermelon from Fusarium wilt, we need sustainable management systems with minimal impact on the environment. Therefore, in this proposal we will attempt to use mycoviruses for the control of Fusarium wilt of watermelon caused by FON.

i. Role of mycoviruses

Mycoviruses are viruses that infect fungi. Generally, viruses have been considered mostly as pathogens that cause diseases in plants, human and animals. However, in this current project, we are exploring viruses as a beneficial microorganism to control fungal growth and important diseases caused by fungal pathogens in plants. The best example is the fungal pathogen *Rosellinia necatrix* and a mycoviruses pathogenic, which forms an interesting pathosystem that attracts scientific and public attention worldwide (Ghabrial and Suzuki, 2009). R. *necatrix* is a phytopathogenic fungi and is the best studied root rot pathogen. R. *necatrix* is a soil-borne fungus that is widespread throughout the world and causes white root rot woody and herbaceous plants.

Mycoviruses are found in major groups of filamentous fungi and an increasing number of novel mycoviruses are being reported (Aoki et al., 2009; Liu et al., 2009; Ghabrial and Suzuki, 2009). Until 1998, no information regarding mycovirus infection in *R. necatrix* was available; however, in 2002, a reovirus was identified from this fungus and since then several novel viruses have been molecularly characterized and include members of at least five virus families.

Successful biocontrol in Europe of the chestnut blight fungal disease using hypovirulent strains inspired a group of Japanese researchers, Dr Matsumoto's group (Matsumoto, 1998) , to conduct an extensive search of a large collection of over 1,000 field fungal isolates of *R. necatrix* for mycoviruses that might serve as virocontrol agents.

Preliminary results obtained previously (by Drs Satoko Kanematsu and Atsuko Sasaki) revealed that dsRNA1 (7.2 kbp) and dsRNA2 (9 kbp) represent the genome segments of a novel bipartite spherical virus (~50 nm in diameter), designated as *Rosellinia necatrix megabirnavirus 1* (RnMBV1). As shown in Fig. 1, RnMBV1 has potential as a virological agent and can be used to control *R. necatrix*, which is the white root rot diseases of economically important fruit and other perennial plants.

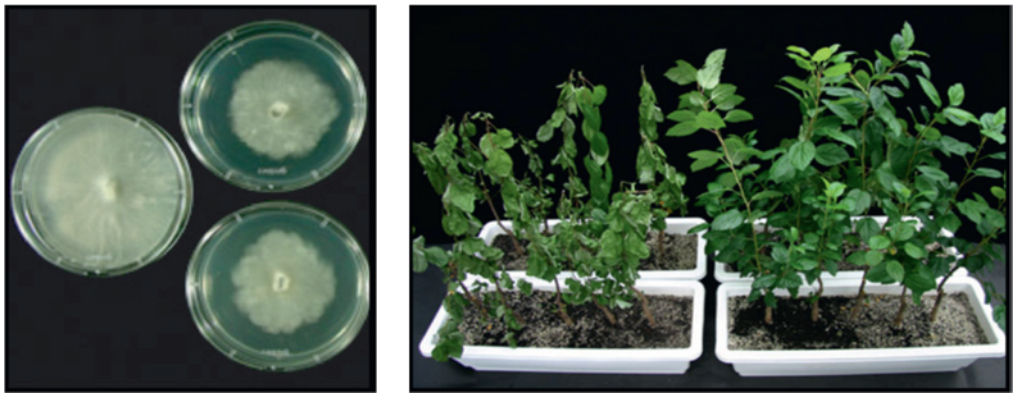


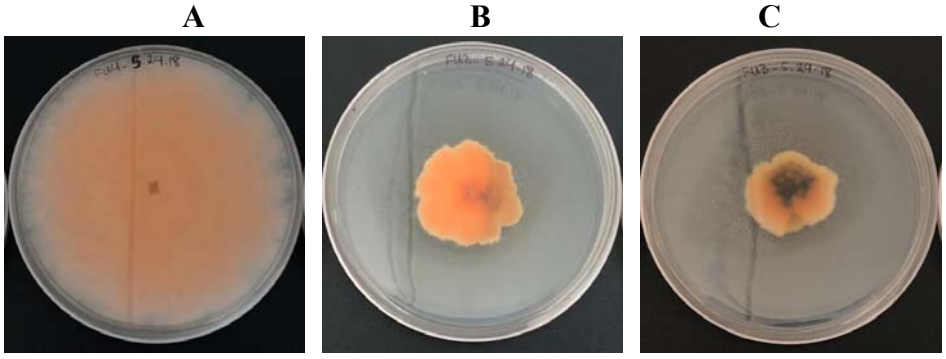
Fig. 1 Colony morphology and virulence of RnMBV1-infected fungal strains. The left panel shows virus-free and RnMBV1-infected colonies cultured on potato dextrose agar (PDA) for 10 days. The right panel shows apple root stocks (10 each) inoculated in the soil with either a virus-free, virulent fungal strain (left) or a RnMBV1-infected hypovirulent fungal strain (right). Inoculated plants were photographed 4 weeks after inoculation Adopted from Chiba et al. (2009).

ii. Current research on mycoviruses in the PI lab

The PI (Dr. Ali) visited Japan last year as a US Fulbright fellow and worked there on mycoviruses in Dr. N. Suzuki lab (one of the leading mycovirologists in the world). Using this experience, the PI has started working on mycoviruses of Fusarium species.

Recently, we have detected hypvoirulent mycoviruses in *Fusarium equiseti* (Fig. 2B-C) that causes Fusarium wilt of Cumin. We have observed phenotypic effects on the growth of fungus on potato dextrose agar media in Petridis's (Fig. 2A-C). We are in the process of characterizing these mycoviruses.

Fig.2



B. Research goals and objectives

The long term goal of this research is to better protect watermelon crops from the deleterious effects of Fusarium wilt and thus improve the reliability of watermelon production. The specific objectives of this research project are listed below:

1. Collection of Fusarium isolates from watermelon

2. Screening of Fusarium isolates for mycoviruses and their characterization

3. Assess the effects of mycoviruses on the growth of Fusarium species for the control of Fusarium wilt in watermelon

C. Experimental procedures

1. Collection of Fusarium isolates

FON isolates will be collected from watermelon fields in selected counties in Oklahoma and Texas by the Principal Investigator. In addition, the project collaborators (see attached letters and section 7 below) will also send FON isolates from different states. The PI has obtained USDA permits for the transportation of Fusarium species all over the United States.

2. Screening of FON isolates for mycoviruses

We will grow all the isolates on potato dextrose agar (PDA) media to produce mycelia. To confirm the presence of an RNA virus in the collected isolates, we will use a dsRNA procedure (already optimized and available in the PI-Lab)to extract dsRNA from fungal mycelia because the genomes of many mycoviruses can be detected in their dsRNA forms. The dsRNA will be run on agarose gel electrophoresis to determine whether a particular FON isolate contain a dsRNA virus or not.

i. Characterization of mycoviruses

Any FON isolates that contain a mycovirus will be further characterized to determine the biological and molecular properties using reverse transcription polymerase chain reaction (RT-PCR), cloning and sequencing.

ii. Phenotypic effects of mycovirus on FON

FON isolates infected with a mycovirus and those that are virus free will be grown on PDA. Effects on growth of the fungus will be recorded and compared with a virus free culture in order to determine a hypovirulent strain of FON.

3. Assess the effects of mycoviruses and pathogenicity test on watermelon seedlings

i. Watermelon seedlings

Seeds of watermelon cultivars will be grown in plastic pots in the growth chamber. One to two weeks old watermelon seedlings will be inoculated with virus-infected and virus-free mycelia of FON isolates. Inoculated plants will be kept in the growth chambers and observed for the development of Fusarium wilt weekly. Data between inoculated and un-inoculated plants will be compared and the effects of hypovirulence on the development of Fusarium wilt of watermelon will be recorded.

ii. Future direction
Based on the experimental results, the experiment will be repeated in the field condition at the Bixby research station to determine the effects of hypovirulence on the development of Fusarium wilt on watermelon.

D. Literature cited

1. Aoki, N., H. Moriyama, M. Kodama, T. Arie, T. Teraoka, and T. Fukuhara. 2009. A novel mycovirus associated with four double-stranded RNAs affects host fungal growth in *Alternaria alternata*. Virus Research, 140: 179–187.
2. Chiba S, Salaipeth L, Lin YH, Sasaki A, Kanematsu S, Suzuki N. (2009). A novel bipartite double-stranded RNA Mycovirus from the white root rot Fungus Rosellinia necatrix: molecular and biological characterization, taxonomic considerations, and potential for biological control. Journal of Virology 83:12801-12812.
3. Ghabrial, S. A., and N. Suzuki. 2009. Viruses of plant pathogenic fungi. Annual Review of Phytopathology, 47: 353–384.
4. Keinath, A. P., and Hassell, R. L. 2014. Control of Fusarium wilt of watermelon by grafting onto bottlegourd or interspecific hybrid squash despite colonization of rootstocks by *Fusarium*. Plant Dis. 98:255-266.
5. Liu, H., Y. Fu, D. Jiang, G. Li, J. Xie, Y. Peng, X. Yi, and S. A. Ghabrial. 2009. A novel mycovirus that is related to the human pathogen hepatitis E virus and rubi-like viruses. Journal of Virology, 83: 1981–1991.
6. Matsumoto, N. 1998. Biological control of root diseases with dsRNA based on population structure of pathogenes. Japan Agricultural Research Quarterly, 32: 31-35.

3. Timelines

Start date: March 01, 2019
End date: Feb 28, 2020

4. Objectives/goals of the project

See section “B” in the project details above

5-6. Periodic dates for tasks and final report (results)

Duration	**Tasks**
March-April, 2019	-Recruit undergraduate students -Order required chemicals and reagents
May-June, 2019	-Collect Fusarium isolates from project collaborators
June- August, 2019	-Collect Fusarium isolates from watermelon fields in Oklahoma and Texas
August-October, 2019	-growing and screening of Fusarium isolates for mycoviruses -Identification of mycoviruses
Nov-Jan 2020	-Pathogenicity test on watermelon seedlings
Feb 28, 2020	-Project final report to NWA

7. Amount of money requested

Funds are requested for necessary supplies, genome sequencing, and for limited travel to collect samples in Florida.

Itemized budget	**Estimated cost**
PI salary	$ 0
Salary for undergraduate students	$ 3,600
Supplies-disposable plastic wares, seeds, tips, soil mix	$ 3,000
Reagents (RT-PCR, cloning, primers)	$ 5,000
Sequencing costs	$ 5,000
Travel to collect samples	$ 3,000
Total from NWA	**$ 19,600**

Matching funds: TU will contribute salary, fringe benefits, and associated indirect costs equivalent to 5% of Dr. Ali's Academic Year time. TU will also contribute unrecovered indirect costs at TU's federally negotiated rate of 40% of modified total direct costs.

Matching funds	**Estimated cost**
PI salary and fringe benefits (36%) at 5% of academic year time	$ 5,107
Contributed indirect costs (for contributed effort above)	$ 2,043
Unrecovered indirect costs on NWA funds	$ 7,840
Total cost share/match	**Up to $ 14,990**

8. List of researchers involved in this project

The PI shall collect Fusarium isolates within Oklahoma and Texas by himself, and with the support of other researchers, isolates from other states shall be collected as well (listed in the Table below). The cooperation and expert advice of these collaborators during this project will be invaluable.

List of other researchers/collaborators involved

Names	**Institutes**	**Funds distribution**
Dr. Akhtar Ali	University of Tulsa	**$ 19,600**
Dr. Kathryn Everts	University of Maryland	0
Dr. Charlie Biles	East Central University	0
Dr. Benny D. Bruton-retired	Former institute-USDA-ARS, Lane , OK	0

NWA priority area

Fusarium wilt

9. Addendum to proposal

Plant pathogens continuously threaten our food crops and thus the world food supply. The total population of the world is predicted to be 8 billion by 2030 and 9 billion by 2050. We need a continuous and growing supply of food to be able to fulfill the needs of a growing population.

Traditionally, viruses have been considered mainly as pathogens that cause diseases in plants, humans and animals. However, in this current project, we are exploring viruses of fungi called mycoviruses (which are not pathogen of plants) as a beneficial microorganism to control fungal growth and important diseases caused by a fungal pathogen in watermelon plants.

Several studies have shown that mycoviruses had adverse effects on the growth of fungi and can be used as a biological control of fungi. Mycoviruses may not have visible symptoms on their host, but they effect the virulence of fungi, which shows how fungi can infect the host, and mostly illustrates itself as hypovirulence. Hypovirulence refers to decreasing the infectious symptom of fungi on its host when the fungi is affected with a mycovirus.

Viruses can be used to control fungal diseases and have been a driving force in mycovirus research since the earliest days. Viruses in the family *Hypoviridae* associated with reduced virulence (hypovirulence) of the chestnut blight fungus, *Cryphonectria parasitica*, is a great example in fungal virology. This has been due in part to the severity of the chestnut blight epidemics in North America and Europe and early reports of hypovirulence-mediated mitigation of disease in European forests and successful application for control of chestnut blight in chestnut orchards. Recently, a more contributing factor has been the development of a hypovirus and *C. parasitica* experimental system that has overcome many of the challenges associated with mycovirus research.

Our proposed project has the potential to identify various mycoviruses from *Fusarium oxysporum* f. sp. *niveum* (FON) isolates, which could have hypovirulence against Fusarium wilt. Using mycoviruses on fungi give this opportunity to use them as a biocontrol instead of fungicide, which are hazardous and dangerous to human health and the environment. They can be used to control the effect of fungi on a host.

At the completion of this project, we will know the hypovirulent FON isolates that will have the potential to biologically control Fusarium wilt in watermelon. This hypovirulent culture can be grown and inoculated into soil for the control of other FON isolates. This strategy will enable us to formulate long lasting control strategies, which are environmentally friendly, efficient against a particular fungus, and economical.

In the last few years, studies of viruses that infect phytopathogenic fungi are gaining due importance because of the current convincing evidence that mycoviruses are responsible for debilitation and/or hypovirulence phenotypes in many economically important plant pathogens. From agricultural perspectives, mycoviruses may contribute to sustainable agriculture as biological control agents. Presently, control of plant pathogenic fungi is a formidable task because of the lack of appropriate disease control strategies. In addition to the health hazards and the risks to the environment, the use of fungicides is often cost prohibitive.

In summary, the more we know about fungal viruses, the more we discredit the idea that all viruses are evil. Mycoviruses are indeed the good guys and hold great promise for exploitation as biological control agents and at the same time they are not pathogen of host plants.

UNIVERSITY OF MARYLAND

Kathryne L. Everts
Department of Plant Science and Landscape Architecture

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(phone) 410-742-8788 (fax) 410-742-1922
keverts@umd.edu

August 28, 2018

Dear Dr. Akhtar Ali:

Please accept this letter in support of your project proposal titled "Mycoviruses of Fusarium wilt of watermelon." As we have discussed previously, I will provide several isolates of *Fusarium oxysporum* f. sp. *niveum* to you, for use in this study. These isolates were collected from the watermelon acreage in the mid-Atlantic region of the U.S., from the states of Maryland and Delaware.

Our watermelon production region has had a long history of infestation by *F. oxysporum* f. sp. *niveum* and with Fusarium wilt on watermelon. The pathogen has been resident here for many years. Fusarium wilt remains an endemic disease in our region and throughout the Eastern U.S. I am looking forward to the results of your research into mycoviruses of *F. oxysporum* f. sp. *niveum*.

Sincerely,

Kathryne L. Everts
Professor, Vegetable Plant Pathology

East Central
UNIVERSITY

1100 E. 14th Street, ~ Ada, OK 74820-6999
580-559-5498 ~ 580- 559-5606 FAX

Dr. Charles L. Biles, Biology

Aug 28, 2018

Akhtar Ali, PhD
Associate Professor
Department of Biological Science
The University of Tulsa, 800 S Tucker Dr
Tulsa, OK 74104-3189

Dear Akhtar
This letter is to express my support and assistance to this project proposal entitled "Suppression of Fusarium wilt of watermelon by mycoviruses" that you will submit to NWA.
I have worked on diseases of watermelon and other cucurbits for almost 30 years. As a results, I have considerable experience in watermelon culture and diseases identification. I maintain communication with watermelon growers, researchers and extension people in the US and generally have good knowledge of problems as they arise over the season.
I have worked with Dr. Ali before in regard to an INBRE collaborative grant where we proposed investigating the presence of dsRNAs in *Monosporascus cannonballus*, another root rot pathogen of melons. I will be glade to continue this collaboration. I also have a collection of *Fusarium* isolates and will be able to give it to Dr. Ali.
I hope that you will be successful with your proposal and I look forward to working with you on this project.

Sincerely,

Charles L. Biles

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Aug 28, 2018

Akhtar Ali, PhD

Associate Professor

Department of Biological Science

The University of Tulsa, 800 S Tucker Dr

Tulsa, OK 74104-3189

Dear Akhtar:

This letter is to express my support and assistance to this project proposal entitled "Suppression of Fusarium Wilt of Watermelon by Mycoviruses" that you will submit to NWA.

I have worked on diseases of watermelon for more than 30 years. As a result, I have considerable experience in watermelon culture and disease identification. Following retirement from the USDA, I have started a consulting service to assist farmers in managing diseases of cucurbits. I maintain communication with watermelon growers, researchers and extension people in the US and have good knowledge of problems as they arise over the season.

I have worked with Dr. Ali since 2008 assisting collection of watermelon samples from Oklahoma and Texas for virus identification. I will be glad to continue this collaboration that we started in 2008.

Dr. Charlie Biles (East Central State University) and I have a very large collection of *Fusarium oxysporum* f. sp. *nevium* isolates representing all the races that we will share with the project. This should be an outstanding project and I am happy to have an opportunity to work with you.

Sincerely,

BENNY D. BRUTON