MOLECULAR INVESTIGATION OF PHOMA SPP. USING REP-PCR TOOL AS A BIOCONTROL AGENT

PROJECT REFERENCE NUMBER: **46S_MSc_077**

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Keywords

***Phoma* species, REP-PCR, Secondary metabolites, Biocontrol Agents**

Introduction / background

As phytopathogens, *Phoma spp.* is widespread throughout the environment, most commonly found in aquatic soil and water. They can cause diseases in plants, animals and humans. Though causing infectious symptoms in the latter is rare. A ubiquitous mold of the Order Pleosporales, Phoma betae can be found in soil and decaying plant materials throughout the world. The most common particles causing sensitization are airborne spores. A variety of crops and foods were found to contain species of the genus *Phoma,* including potatoes, nuts, bananas, tomatoes, sorghum and maize.

Colony colours range from white to pinkish white or violet. On most media, it grows slowly, but produces an abundance of pycnidia. Pycnidia globular, sub globular to irregular, violet, not carbonaceous, 725-330 x 695-264,µm, ostiolate; Ostiole one, two or more, large, 265-56u in diameter, surrounded by short rows of cells (short filaments) terminating in relatively large, thin-walled cells. Pycnidial cavity solitary; Conidiophores short, simple, translucent, terminally bearing conidia. (V.H. Pawar et al., 2009). Lately, a large number of structurally unique metabolites with implicit natural and pharmacological conditioning have been insulated from the marine *Phoma* species particularly, *Phoma herbarum, P. sorghina* and *P. tropical.* Diterpenes, enolides, lactones, quinine, phthalate, and anthraquinone are the primary components of these metabolites. The majority of these substances have cytotoxic, antibacterial, anticancer, and radical scavenging effects (Mahendra Rai et. al., 2018).

It is likely that technological developments in whole genome sequencing and data analysis in the coming years will facilitate their routine use for population structure, epidemiological studies, and phylogenetic analyses of Phoma species. These are

likely to reveal more minor *Phoma* clades and to enhance our understanding and the analysis of the advantageous properties of this versatile organism

Objectives:

1. Isolation of *Phoma* species (fungi) from diverse sources (marine soil, potato rot sample, leaf sample, etc.).
2. Molecular characterization of native *Phoma* isolates.
3. Screening of cassettes of genes and Gene expression studies by genomic methods for biocontrol activity.
4. Molecular genotyping and functional diversity for novel *Phoma* Species by using REP PCR techniques.
5. Sequential Extraction of secondary metabolites in *Phoma* species and purification of compounds by HPLC, GC-MS and NMR studies.
6. Bioinformatics approach, Data interpretation and Analysis of *Phoma* species
7. Application aspects of above potent isolates by screening for nanoparticles synthesis as a future perspective.
8. Analyzing the genome editing by CRISPR- Cas9 technology in *Phoma spp.*
9. Antimicrobial activity of secondary metabolites against MDR strains
10. Synthesis of nanoparticles or C-quantum dots against pathogenic organisms as application as aspects

Methodology:

1. Metagenomic and Non-Metagenomic approach for the isolation of *Phoma* Sp.

The *Phoma* Sp will be readily isolated from diverse sources (marine soil, potato rot sample, leaf sample, etc.) and can also be cultured in laboratory using various with minor modifications.

For each composite sample, 10 grams of soil will be diluted in 90 ml of sterile, de-ionized, distilled water and agitated for 30 minutes. On Ohio Agar, a soil suspension was serially diluted in ten microlitres (10^{-2} and 10^{-3}) before being incubated at 28° C. (Dhingra and Sinclair, 1995). After 72 hours, chosen fungal colonies were transferred to acidify potato dextrose agar based on morphological traits such dematiaceous mycelia (PDA). At 4°C, fungus isolates were stored on PDA (Ismael E. Badillo Vargas *et. al*., 2008).

2. DNA isolation of *Phoma* Sp.

Total genomic DNA will be extracted and quantified by using (Possiech and Neumann, 1968) with minor modifications.

DNA will be also prepared from freeze-dried mycelium of *Phoma spp*. by the method of (Raeder and Broda,1985) and will involve phenol/chloroform extraction of powdered mycelium in buffer (200mmol/l Tris-HCl, 250mmol/l NaCl, 25mmol/l EDTA, 0·5% w/v SDS pH8·5) and following Proteinase K treatment of powdered mycelium in buffer (50mmol/l Tris-HCl, 150mmol/l EDTA, 1% w/v sodium lauryl sarcosine pH8·0).

3. PCR amplification of conserved universal gene (18s rRNA, ITS region)

18srRNA will be amplified by using universal primers ITS 1 and ITS 3.

In a 150 ml Erlenmeyer flask containing 20 ml of PD broth medium, the discs of two agar plugs (5 mm in diameter) from the borders of actively growing hyphae of the test fungus will be injected. Five days of culture maintenance on a rotating shaker at a temperature of 25 2 °C will be followed by harvesting by centrifugation at 5000 g for ten minutes. Following two water washes, the mycelia will be separated by centrifugation at 3000 g for 15 minutes. Then, a pre-chilled mortar and pestle will be used to grind 1 g of mycelia into a fine powder.

Standard procedures will be used for the PCR amplification and fungal DNA extraction (Kumaran et al., 2011). PCR amplified product will be purified and sent for sequencing for identification of nucleotide sequence and the result will be interpreted with NCBI BLAST Tool.

4. Rep-PCR of *Phoma* Sp.

This technique will be used for the identification of native *Phoma spp*. isolates for identification at species and strain level. The repetitive sequence-based PCR, also known as rep-PCR, DNA fingerprint technique creates distinctive DNA profiles, or "fingerprints," of individual microbial strains using primers that target many of these repeated components.

Primers: REP (REP1RI:5'-IIIICGICGICATCIGGC3' and REP2I:5'-ICGICTTATCIGGCCTAC-3') and ERIC (ERIC1R: 5' ATGTAAGCTCCT GGGGATTCA C-3' and ERIC2: 5'-AAGTAAGTGACTGGGGTGAGCG-3') primers were described by Versalovic et al. (1991); the sequence of the BOX primer (BOX A1R: 5'-CTACGGCAAGGCGACGCTGACG-3') was described by Versalovic et al. (1994).

Conditions for PCR: 25 ng of DNA, 200 M each of dATP, dGTP, CTP, and TTP, 1.30 mM each of the primers, and 1 unit of Taq DNA polymerase (Appligene) were all present in the 25µl reaction mix in a 0.5-ml microtube. The Perkin-Elmer DNA Thermal Cycler 480 was used to carry out the PCR amplifications. The PCR settings were as follows: I for LMR1-based PCR, 30 cycles of 1 min each at 94°C, 52°C or 60°C, and

72°C; (ii) for REP and ERIC, cycles specified by Versalovic et al. (1991); and (iii) for BOX, cycles described by Versalovic et al (1994). Annealing temperatures for REPPCR, ERIC-PCR, and BOX-PCR were 40°C, 52°C, and 53°C, respectively (Malgorzata Jedryczka et. al., 1999)

5. Sequential extraction of secondary metabolites in *Phoma* species

This method will be used for the sequential extraction of genuine compounds using polar and non-polar solvents. Further, purification using new advanced techniques like HPLC, GC-MS, GC-MS/MS, NMR, FTIR etc.

The amount of secondary metabolites produced by microorganisms under different growth circumstances can be estimated using high-performance liquid chromatography (HPLC) analysis of *Phoma* isolate extracts, however the process is slow (Richard E. Higgs et.al.,2001). Although there are significant examples of the use of surface enhanced Raman spectroscopy and Fourier-transform infrared spectroscopy (FTIR), metabolomics has generally relied on the independent use of mass spectrometry (MS) or nuclear magnetic resonance spectroscopy (NMR) (Fatema Bhinderwala et. al., 2018).

6. Bioinformatics approach, Data interpretation and Analysis of *Phoma* species

Evolutionary and phylogenetic distances and relatedness of the native isolates can be fingerprinted and identified for renowned strains in terms of functional activity. The goal of BLAST is to identify any local regions (of either amino acids or nucleotides) where two sequences are similar. One of the two sequences is the unknown when BLAST is used to identify species, and the other sequence is often taken from sizable public databases of genetic sequences.

7. Application aspects of above potent isolates by screening for nanoparticles synthesis as a future perspective.

The microbe will be cultivated in fluid Czapek Dox broth with 21 grams of sucrose and 3 grams of yeast extract in 1 liter of distilled water. It was then incubated for seven days at 28 degrees Celsius and 200 revolutions per minute (IKA KS 4000). The culture will be centrifuged for five minutes at 10,000 rpm, and the supernatant was then used to create silver nanoparticles. In order to make a silver nitrate solution (0.1mM), 0.017 g of the substance was dissolved in 100 ml of double-distilled water. After that, 100 ml of the supernatant and 100 ml of a silver nitrate solution at a concentration of 1 mM were combined, and they were incubated once again for 24 hours at 28°C (the total concentration of silver nitrate was then 0.5 mM). The colors of the solutions changed to dark buff, indicating the formation of silver nanoparticles in the solution. The solutions will be centrifuged at 1,000 g, the particles separated from the supernatant and dried in 40 °C. Finally, the silver nanoparticles will be stored carefully in dark vials for further analysis (Soheyla Honary et.al. 2012). *P. herbarum* is one of the fungi that

contain brefeldin A, a substance with strong phytotoxic properties. It is a macrocyclic lactone with a wide range of biological effects, including cytotoxicity, antifungal, and antiviral. Brefeldin A inhibits intracellular protein export, having a significant impact on the Golgi apparatus's structure and operation in animal cells, according to Betina (1992). (Cole et al., 2000). Commercially available antimicrobial discs will be used for Biocontrol assays from the isolated strains. For validation, MIC test will be evaluated.

8. Analyzing the genome editing by CRISPR- Cas9 technology in *Phoma spp.*

The enhanced CRISPR systems, like CRISPR/CRISPR-associated protein (Cas)9 and CRISPR/Cpf1, can offer a viable new route for creating beneficial and promising variations that are resistant to many diseases. The entire genome sequences of various plant species and the improvements in biotechnological techniques will surely increase the precision of breeding for continued disease resistance.

Results and Conclusions

It is likely that technological developments in whole genome sequencing and data analysis in coming years will facilitate its routine use for population structure, epidemiological investigations, and phylogenetic analyses of Phoma species. These are likely to reveal more minor *Phoma spp.* clades and to enhance our understanding of the population biology of this versatile organism. When the primers inferred from Phoma gene intron are used, these PCR approaches would be very beneficial from an analytical and therapeutic point of view and will be helpful for epidemiological and taxonomic issues. Phoma-related fungi frequently grow on economically significant crop plants, where they cause fatal plant diseases. Crop plants are attacked by pathogenic *Phoma sensu lato* species members who display symptoms ranging from leaf blight to root rot and even plant wilting. So, we can eradicate such detrimental diseases by introducing biocontrol activities in the following context.

What is the innovation in the project?

Recently, green synthesis of metal nanoparticles by the application of fungi has gained special investigation due to protect ecosystem. Different AuNPs applications are as bio-sensor, drug-delivery, bio-imaging, catalysts, gene delivery, antimicrobial, tumor imaging and bio-assay. Currently Fungi have been applied for the biosynthesis of the metal-based nanoparticles. Therefore, fungi have more advantages than bacteria for bioprocessing, involving green synthesis of gold nanoparticles. The so-called secretome comprise all of the secreted proteins into the extracellular space. Many fungal secretome concentrations have been used for huge for producing proteins and the extracellular proteins and enzymes play a vital role in Au reduction and AuNP capping. Having high metal toxicity resistance is the benefits of using fungi for synthesis of gold nanoparticles. AuNP biosynthetic potentials in different bacteria and fungi suggest that the diminish of Au^{3+} to form protein metal nanoconjugates is a common response to toxic stress, where the enzymatic machinery needed is readily

available in environmental microorganisms. Several studies on biosynthesis of AuNPs by the soluble protein extract of *Fusarium oxysporum* released that Nicotinamide adenine dinucleotide hydrogen (NADH) -dependent reductases are involved in the bioreduction process. The antibacterial effectiveness of AuNPs was related to the size and dispersibility of nanoparticles. The smaller AuNPs in diameter is more applied in tissue immunology, biochemistry and high-powered microscopy. In environmental research, DNA testing and drug delivery, medium-sized AuNPs are mostly used. Larger AuNPs are used in medical, electrical and X-ray optics. The mechanism for antibacterial demonstrated by the biosynthesized AuNPs is subjected to the various degree of susceptibility of bacteria. When the AuNPs come in contact with the microbe, it binds with the bacterial surface through electrostatic interaction. The distinct smaller size and proton motive force of the synthesized AuNPs enable their penetration into the bacterial cell through the membrane proteins. The (AuNPs) have shown uniquely advantageous antimicrobial properties. As the least active metal, gold has very stable chemical activities, is non-toxic and has well biocompatibility. Compared with gram-positive bacteria, the structure of peptidoglycan cell walls, which gram-negative bacteria have, may cause differences in the antibacterial effect of AuNPs

Scope for future work

The use of synthetic fungicides to control fungal diseases has growing limitations due to eco-toxicological risks. Therefore, it is necessary to replace or integrate high risk chemicals with safer tools for human health and environment. Consequently, research on the selection, evaluation, characterization, and use of biocontrol agents (BCAs) has consistently increased in the last decades. BCA formulates, particularly in some countries, are still scarce in coping with the growing demand for their use in sustainable agricultural management. To foster development and utilization of new effective bioformulates, there is a need to optimize BCA activity, to share knowledge on their formulation processes and to simplify the registration procedures. Studies based on new molecular tools can significantly contribute to achieve such objectives. Thus, **the present scope for future work** includes the state of the art on biocontrol of fungal plant diseases with special emphasis on (i) features of the most studied BCAs; (ii) key strategies to optimize selection and use of BCAs (iii); mechanisms of action of the main BCAs; (iv) molecular tools and metagenomic studies in the selection and use of BCAs; (v) main issues and constraints in the registration and commercialization of BCAs, and (vi) perspectives in the biocontrol of fungal plant diseases. On the other hand, the growing demand for agri-food products due to the steady increase of the world population is causing an urgent need for new effective control tools/strategies. These should be capable of integrating, or even replacing, synthetic pesticides so that high production standards and higher sustainability in agricultural production are ensured. Among natural products, beneficial microorganisms (biocontrol and/or plant bio-stimulant microorganisms) appear to be the most promising tools to ensure plant health, as well as quality and safety of vegetal products.