Management of *Macrophomina phaseolina* causing charcoal rot disease in soybean crop

A Summary submitted to the
Babasaheb Bhimrao Ambedkar University, Lucknow
in fulfilment of Requirement for the Award of Degree of

Doctor of Philosophy

in Environmental Science

BY

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Enrolment No. 1118/15

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2023

Summary

Soybean is one of the most valuable legume crops of the Fabaceae family and is commonly known as a cash crop. It grows well in hot and humid climates throughout the globe. India has fifth ranks in terms of area and production of soybean globally. Soybean is one of the cheapest and richest source of protein, carbohydrates, oil, vitamins (A, B, C, E) and minerals. So, it is known as a poor man's meat or a staple diet for people and animals in many parts of the world. Soybean is a commercial crop worldwide and production is increasing as per of demand of food. The use of soybean can provide many health benefits such as the prevention of heart disease, regulation of blood cholesterol and blood pressure, immune disorders and kidney-related problems. The soybean plant also plays an important role in the conversion of atmospheric nitrogen into organic forms which is an essential plant nutrient that eventually influences crop output, reduces the cost of crop production and enhances the economic feasibility of soybean for the production of different products. Soybean cultivation areas and production are increasing year by year across the world. The top five soybean producing countries in the world are Brazil, the U.S., Argentina, China and India. In 2021–2022, the total global soybean production was 385.524 metric tons. In India, the total sowing area was 114.487 million hectares and the total estimated production was 120.383 million metric tons as per the report of SOPA 2022.

Soybean is susceptible to yield loss due to abiotic (Temperature, rainfall, drought, etc.) and biotic factors (fungi, bacteria, viruses and nematodes) that affect the yield, seed viability, as well as the nutritional value of the crop. Among them, the most yield loss occurs, especially through *Macrophomina phaseolina***,** a ubiquitous, necrotrophic, thermophilic fungus that causes charcoal rot disease in soybean crop. The

fungal pathogen can infect soybean crops at any stage of development. The first indications of charcoal rot in the soybean crop are reduced vigour, yellowing of leaves and brown to red staining on stems and roots followed by wilting and premature senescence, ultimately leading to plant death.

The agricultural industry is experiencing several challenges in managing various diseases caused by fungal infections. Moreover, the increasing resistance of fungal pathogens against chemical pesticides is further increasing the problem. So, different methods have been employed to control *M. phaseolina*, but effective management is not available due to the soil-borne nature of the fungus. Biological methods are efficient, cost-effective and environment-friendly way to deal with plant diseases. Moreover, soil-borne diseases have been controlled by using beneficial microbes isolated from the rhizospheric zone of plants. Bio-agents can help to elevate crop production and serve as a sustainable means to control disease and reduce dependence on synthetic chemicals. Previously, different species of micro-organisms such as *Aspergillus*, *Trichoderma* and *Penicillium* have been used to control plant disease. *Trichoderma sp.* is a rhizospheric, mycoparasitic that protects plants from soil-borne pathogens through the mechanisms of competition, antibiosis, or mycoparasitism. *Trichoderma sp.* destroys the microsclerotia through the processes of coiling, penetration and lysis and absorbs nutrients for its own growth in the soil. The secondary metabolites secreted by the fungus also play a considerable role in crop disease management. Likewise, plants or their parts have also been used since ancient times in Indian society for the management of different types of diseases due to the presence of different phytochemicals (secondary metabolites) that have a potent ability to manage plant pathogens. The secondary metabolites are the different groups of natural compounds. such as alkaloids, flavonoids, tannins, saponins, linoleic acid and

anthraquinones, which have the potential to show antifungal activity. Overall, plants and microorganisms have natural substances that can play a considerable role in sustainable disease management. Considering the role of antagonists and plant extracts in controlling the growth of *Macrophomina phaseolina* economically and sustainably.

Therefore, in the present study, we have performed the following work:

- Collection of infected and healthy soybean plants with intact rhizospheric soil.
- Isolation and identification of mycoflora from the rhizospheric soil of the healthy plants and infected plants.
- Antagonistic activity of some selected bioagents against *M. phaseolina* under *in vitro* condition.
- Antifungal activity of some selected medicinal plants extracts against *M. phaseolina* under *in vitro* condition.
- Study of the combined activity of selected bioagents and extract of medicinal plant against *M. phaseolina* under in-vitro and *in vivo* conditions.

❖ **The key finding of the entire study after critical evaluation and interpretation of the experimental data are as follows:**

4.1. Isolation and identification of pathogen

The isolated pathogen from infected parts of soybean was identified as *Macrophomina phaseolina* Gmax20. The isolated pathogen showed fast growth and covered the entire plate within 3 days and turned dark black in colour within 7 days.

4.2. Pathogenicity test and histopathological study

The pathogenicity test of *M. phaseolina* was performed by following Koch's postulate method. The results showed that the symptoms of the disease such as small black dots shaped microsclerotia beneath the epidermis layer of plants after 30 days of sowing in soil inoculated with *M. phaseolina*. The diseased part was transferred to the PDA plate and morphological characteristics of the re-isolate were compared with the previous isolate (*M. phaseolina*) and the results were found similar. The histopathological study of infected stems (T.S. and L.S.) under compound microscope and SEM at different magnifications revealed that xylem vessels were ruptured due to penetration of fungal hyphae. Finally, the results showed that the fungus infection spreads from the roots to the aerial parts of the plant and ultimately causes the wilting of the entire plant.

4.3. Isolation and identification of antagonistic mycoflora

In the present study, 12 fungal isolates were tested for antagonistic activity against M. phaseolina, 12 fungus isolated from the rhizospheric soil soybean plants.in which only five fungal strains (*Trichoderma isolate Gmaxr1 and Gmaxr2, A. niger, A. flavus* and *A. fumigatus*) showed antagonistic potential in the dual plate method.

4.4. Molecular identification

Molecular characterization is essential for accurate identification of *Trichoderma* spp., because morphological signs are misleading. In this study, the 18SrDNA sequencing technique was used for the genomic identification of the fungal pathogen isolate Gmax20 and antagonist *Trichoderma* Gmaxr1 and Gmaxr2 and compared it with the entire microbial genome database at NCBI (National Center for Biotechnology Information). The sequences of isolates were deposited in GenBank under the names of *M. phaseolina* Gmax20 with accession number OM004744 whereas

Trichoderma crassum Gmaxr1 with accession number OM638646 and *Trichoderma regulosum* Gmaxr2 with accession number OM638647.

4.5. Antagonistic activity of some selected rhizospheric isolates against the *M. phaseolina* Gmax20 by dual culture technique

The antagonistic ability of all isolated rhizospheric fungi were tested, among them only four species namely *Trichoderma crassum* Gmaxr1, *Aspergillus niger*, *Aspergillus flavus*, *Aspergillus fumigatus* showed optimistic outcome against the pathogenic fungus *Macrophomina phaseolina* Gmax20. The *T. crassum* Gmaxr1 showed the highest inhibition on 1st day (16.98%), 2nd day (23.08%) and 3rd day (54.04%) against *M. phaseolina* Gmax20. However, *A. niger* showed least inhibition 15.09%, 15.38% and 33.84% on the 1stday, 2nd day and 3rd day respectively. In the present study, the promising ability of *T. crassum* Gmaxr1 to control *M. phaseolina* could be attributed to competition and antibiosis.

4.6. The mycoparasitic activity of *T. regulosum* gmaxr2 against *M. phaseolina* Gmax20

In the present study, *T. rugulosum* Gmaxr2 showed mycoparasitic activity and completely reduced the hyphal growth of *M. phaseolina* Gmax20 on the 3rd day after and started growing upon the pathogen. The outcomes showed that *T. rugulosum* Gmaxr2 restricted the growth of *M. phaseolina* Gmax20 by 11.32%, 36.75%, and 50.52% on the 1st, 2nd and 3rd day respectively. The finding of this study epitomizes that *T. rugulosum* Gmaxr2 showed remarkable potential in controlling the growth of *M. phaseolina* Gmax20.

4.6.1. Compound light microscopy

The results showed that within 3 days of incubation on PDA Petri plates, *T. rugulosum* Gmaxr2 established hyphal attachment to *M. phaseolina* Gmax20. The mycoparasitic activities along with physical and morphological changes were observed, such as the overgrowth of *T. rugulosum* Gmaxr2 hyphae on the hyphae of *M. phaseolina* Gmax20.

4.6.2. Scanning electron microscope

The SEM images depicted that the hyphae of *T. regulosum* Gamxr2 first established physical contact with the hyphae of the pathogen and then started growing in parallel and penetrated in the host hyphae with the help of haustoria also showed the deformation of the hyphae of the pathogen after the penetration of antagonist hyphae.

4.7. Impact of *T. crassum* Gamxr1 and *T. regulosum* Gamxr2 on *M. phaseolina* Gmax20 through food poison technique

Extract of *Trichoderma crassum* Gamxr1 possessed antifungal activity against *M. phaseolina* Gmax20 and it controlled the growth by 84.08 % at 75% v/v concentration followed by 35.37%, 59.94%, 68.78% at 10%, 25%, 50% v/v concentration respectively on 3rd day under *in vitro* condition. *Trichoderma regulosum* Gamxr2 inhibited the growth by 100% at 75% v/v concentration followed by 45%, 62.11% and 74.76% at 10%, 25%, and 50% v/v concentration against *M. phaseolina* Gmax20 on 3rd day after treatment under *in vitro* condition.

4.8. Gas chromatography-mass spectrometry (GC-MS) analysis of *T. crassum* gmaxr1 and *T. regulosum* gmaxr2

The chromatogram of GCMS analysis of *T. crassum* Gmaxr1 and *T. regulosum* Gmaxr2 showed the peak of various compounds. The bioactive compounds detected in

the extract of *T. crassum* Gmaxr1 and *T. regulosum* Gmaxr2 and responsible for the antifungal and antimicrobial activity at different levels.

4.8.1. GC-MS analysis of extract of *T. crassum* Gmaxr1

The GCMS analysis of *T. crassum* Gmaxr1 In ethyl acetate and chloroform fraction and various compounds are detected. The major compounds detected in the ethyl acetate fraction of *T. crassum* Gmaxr1 were Diethyl Phthalate (5.61%), Hexadecanoic acid, methyl ester (6.88%), 9-octadecenoic acid (z)-, methyl ester (11.13%), Glycidyl palmitate (7.35%), 9-octadecenamide (4.27%), 9-Octadecenoic acid (Z)-, oxiranylmethyl ester (9.97%), Linoleyl pentadecylate (4.82%).

In chloroform fraction of *T. crassum* Gmaxr1**,** Hexadecanoic acid, methyl ester (8.51%), 9,12-Octadecadienoic Acid (Z, Z)-, ,9-Octadecenoic Acid (Z)-, Methyl Ester (13.68%), Hexadecanamide (3.44%), Glycidyl palmitate (9.41%), 9-Octadecenamide, (Z)-(4.82%), 9-Octadecenoic acid (Z)-, oxiranylmethyl ester (15.11%), 16-Hentriacontanone (2.78%), Phenol, 2,4-bis (1,1-dimethylethyl)-, phosphite (3.13%), Erucyl 11-cis-eicosenoate (9.28%).

4.8.2. GC-MS analysis of extract of *T. regulosum* Gmaxr2

The chromatograph and detected compounds of ethyl acetate and chloroform fraction of *T. regulosum* Gmaxr2 extract. Major privilege compounds of ethyl acetate fraction as, Trifluoroacetic acid (7.04%), Hexadecanoic acid, methyl ester (6.01%), n-Hexadecanoic acid (15.73%), 9-Octadecenoic Acid (Z)-, Methyl Ester (9.91%), 9-Octadecenoic acid(5.99%), Hexadecanamide(2.80%), Glycidyl palmitate (5.26 %),9-Octadecenamide(3.58%), 9-Octadecenoic acid (Z)-, oxiranylmethyl ester (7.06%), Linoleyl palmitate (5.32%), Phenol, 2,4-bis (1,1-dimethylethyl), phosphite (4.27%), (Z)-(Z)-Docos-13-en-1-yl icos-11-enoate (6.58%).

Chloroform fraction of *T. regulosum* Gmaxr2 as, Methane, Sulfinylbis (4.60%), Hexadecanoic acid, methyl ester (6.33%), n-Hexadecanoic acid (23.36%), 9-Octadecenoic Acid (Z)-, Methyl Ester (10.41%), 9-Octadecenoic acid (4.61%), Glycidyl palmitate (4.24%),9-Octadecenamide (3.38%),9-Octadecenoic acid (Z)-, oxiranylmethyl ester (5.52), Tris(2,4-di-tert-butylphenyl) phosphate (3.38%), Linoleyl palmitate (4.88%), Erucyl 11-cis-eicosenoate (6.07%).

4.9. Effectiveness of different plant extracts against *Macrophomina phaseolina* Gmax20

Among all examined medicinal plant and fungus extracts, *D. stramonium*, *T. regulosum* Gmaxr2 and mixed extract of *D. stramonium* + *T. crassum* Gamxr1 + *T. regulosum* Gmaxr2 completely (100%) inhibited the growth of *M. phaseolina* Gmax20 at 70% v/v concentration on 3rd day incubation under invitro condition. *D. stramonium*, *T. regulosum* Gmaxr2 and mixed extract of *D. stramonium* + *T. crassum* Gamxr1 + *T. regulosum* Gmaxr2 had the best inhibition potency to control *M. phaseolina* Gmax20 in comparison to *A. indica, Ocimum sanctum, C. gigantea, T. cordifolia* and *T. crassum* Gmaxr1.

4.10. Effect of *T. regulosum* Gmaxr2 and *D. stramonium* against *M. phaseolina* Gmax20 under *in vivo* condition

D. stramonium, *T. regulosum* Gmaxr2 and *D. stramonium* + *T. regulosum* were used against the charcoal rot-causing fungus *M. phaseolina* Gmax20 in vivo condition. The disease incidence in the soybean plant was found 0% with an inoculum of *T. regulosum* Gmaxr2, and *D. stramonium* + *T. regulosum* Gmaxr2 at 4% w/w concentration and 100% healthy.

T. regulosum Gmaxr2 and *D. stramonium* + *T. regulosum* showed the best performance to control the disease incidence and enhanced vegetative growth of soybean plant in comparison to *D. stramonium*