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Mycoparasitic Study of *Trichoderma rugulosum* Gmaxr2 against *Macrophomina phaseolina* Gmax20 Causing Charcoal rot in Soybean Crop

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ABSTRACT

Soybean is frequently grown as a cash crop to assist farmers in improving their financial status. Charcoal rot is one of the most serious diseases of soybean crop. Therefore, the management of this disease is of utmost importance for enhanced production of soybean. In the present study, the samples for the isolation of pathogen and antagonist were collected from the soybean field. The isolated fungal isolate Gmax20 and Gmaxr2 were genetically identified as *Macrophomina phaseolina* Gmax20 and *Trichoderma rugulosum* Gmaxr2 by NCMR Pune. The results depicted that *T. rugulosum* Gmaxr2 reduced the development of *M. phaseolina* Gmax20 by 50.52 % as compared to the control. The mycoparasitic activity of *Trichoderma rugulosum* Gmaxr2 against *Macrophomina phaseolina* Gmax20 was evident from the compound and scanning electron microscope. Overall, the results suggest that *T. rugulosum* Gmaxr2 can be used as an effective biocontrol agent for the management of *M. phaseolina* Gmax20.

Keywords: *Macrophomina phaseolina*, Biocontrol, Mycoparasitism, Charcoal rot, Soybean

INTRODUCTION

Soybean also called a miracle crop is an important source of protein in vegetarian diet[20]. Many products made from soybean are available in the market, such as soy cheese, soya butter, etc.[22]. Soybean is grown in more than 50 countries around the world, with India ranking second in overall production. In India, Madhya Pradesh is the forefront producer of soybean with 60% of the total cultivation area[27]. Like other crops, the production of soybean is reduced due to various abiotic (drought, temperature, rain) and biotic (fungus, bacteria, viruses) factors[3]. Soybean crop is mainly affected by the seed soil born necrotrophic fungus *Macrophomina phaseolina,* which is responsible for charcoal rot disease[1]. 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, premature senescence, and ultimately leading to plant death[6].

Macrophomina phaseolina is an ubiquitous, necrotrophic[25] thermophilic fungus that causes charcoal rot (stem canker), stem blight, and dry weather wilt disease in more than 500 species

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of approximately 100 plant families[6]. Although, it is found around the world but tropical and subtropical countries are more conducive for the germination of sclerotia[12]. The disease was first reported in the United States in 1949[26] and is considered as the most devastating disease in the countries which are leading in soybean production[30]. The fungus exists in two asexual stages; the first is a sclerotial stage called *Rhizoctonia botaticola*, and the second is a pycnidial stage called *Macrophomina phaseolina*[6]. The infection of the *Macrophomina phaseolina* produces a compact structure inside host tissue known as microsclerotia that remains in the soil for several years and acts as a primary source of inoculum. Moreover, the density of sclerotia plays a significant role in the spread of disease[19].

Several physicochemical methods have been used for the management soil borne diseases but being less effective and expensive cannot be widely used for the management of charcoal rot disease. Chemical fungicides often affect non-targeted organisms as well as beneficial soil microflora when consistently applied for the management of plant disease[4]. Furthermore, the residue of fungicides may enter the bodies of water through run off and accumulate in aquatic flora and fauna[2]. Due to multiple drawbacks associated with physico-chemical methods, the paradigm is shifting toward promising biological methods. Biological control methods that utilize natural substances such as plant products and micro-organisms have emerged as a more viable option for the management of plant pathogens.

Several researchers have reported that *Trichoderma* species is a potent bioagent against various plant pathogens[7,10,18]. *Trichoderma* is a rhizospheric, necrotrophic mycoparasite that protects plants from soil-borne pathogens through the mechanisms of competition, antibiosis, or mycoparasitism[29]. In mycoparasitism, *Trichoderma* destroys the sclerotia by the processes of coiling, penetration, and lysis and absorbs nutrients for its own growth in the soil[28]. Mishra and Dantre also observed that the use of secondary metabolites of *Trichoderma* for seed treatment controls the charcoal rot in the field condition[21]. Previously, several species of *Trichoderma* have been observed to potentially control the growth of *Macrophomina phaseolina*. But there is no information available on *Trichoderma rugulosum* as a biocontrol agent against *Macrophomina phaseolina.* Thus, the present study was conducted to identify the species of *Trichoderma* that can effectively hamper the growth of *Macrophomina phaseolina*. The main objective was to examine the mycoparasitic activity of *Trichoderma rugulosum* Gmaxr2 against *Macrophomina phaseolina* Gmax20 through the compound light microscope and scanning electron microscope.

Materials and methods

Sample collection, isolation of pathogen and antagonistic mycoflora

Samples were collected from the infected soybean fields of the district Rewa, Madhya Pradesh for the isolation of pathogen and antagonist. Healthy and infected soybean plants along with rhizospheric soil were collected in the sterile polybags. The pathogen (isolate Gmax20) was isolated from infected parts of soybean plant on the PDA plate. Similarly, the antagonistic mycoflora (isolate Gmaxr2) was isolated from the rhizospheric soil of the soybean plant through the serial dilution method. The isolated pathogen and mycoflora were purely maintained on different PDA plates and kept in a refrigerator at 4˚C for further analysis.

Identification of pathogenic isolate gmax20 and antagonist isolate gmaxr2

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For confirmed identification, isolated fungal isolates Gmax20 and Gmaxr2 were forwarded to NCMR Pune for genetic identification. The molecular identification of both fungus was proceeded by extracting genomic DNA. PCR was done by using universal primers ITS1 and ITS4. The automated DNA sequencer ABI 3730XL (Applied Biosystems, Inc., Foster City, CA) was used to sequence purified PCR products according to manufacturer instructions. The Basic Local Alignment Search Tool (BLAST) of the National Center for Biotechnology Information (NCBI) was used for the identification of sequenced data. The accession number was assigned after the deposition of the ITS region sequence.

Efficacy of isolate gmaxr2 against pathogenic isolate gmax20 by dual plate method

The activity of antagonistic isolate Gmaxr2 against Pathogenic isolate Gmax20 was evaluated by dual plate method under invitro condition. In the dual plate method, PDA media prepared was autoclaved at 121˚C for 30 minutes. The media after cooling was finally poured into sterilized Petri plates. A five mm block of isolate Gmax20 was kept on one side and the antagonist (Gmaxr2) on the opposite side in the same Petri plates with the help of a sterilized cork borer. Similarly, a 5 mm diameter plug of isolate Gmax20 was put on the margin of another Petri plate which served as control. All treated and control plates were incubated in an incubator at 26±2˚C. The mycelia of treated and control plates were measured on the 1st, 2nd, and 3rd day. The inhibition percentage of isolate Gmax20 was calculated by the following formula:

$$\textbf{Inhibition}\% = \frac{\textbf{Control} - \textbf{Treatment}}{\textbf{Control}} \times 100$$

The mycoparasitic activity of isolate gmaxr2 against isolate gmax20

For mycoparasitic activity, the hyphal interaction of Isolate Gmaxr2 against isolate Gmax20 was studied using the dual culture plate method. The mycoparasitic activity began after 3 to 4 days as the antagonistic isolate Gmaxr2 started to grow on the pathogen (Gmax20) mycelium. The mycoparasitic activity was studied using Compound light and Scanning electron microscope.

Compound light microscopy

Cellophane tape technique was used to observe the interaction between the antagonist and the pathogen. A piece of cellophane tape was put in the interaction zone of a 3–5-day old culture of the antagonist isolate Gmaxr2 and pathogenic isolate Gmax20, and gently pressed with the help of a brush. After this, the cellophane was stuck on the slide containing cotton blue. The prepared slide was examined under the compound light microscope at various magnifications.

Scanning electron microscope

The sample was prepared according to the method of Mycock and Berjak[24]. Ten mm diameter plug was cut from the 3 to 5day old hyphal interaction between the isolate Gmaxr2 and Gmax20. The prepared sample was examined using a research-grade SEM (Scanning electron microscope, etc.). Various magnification levels were used to take electron micrographs of the sample.

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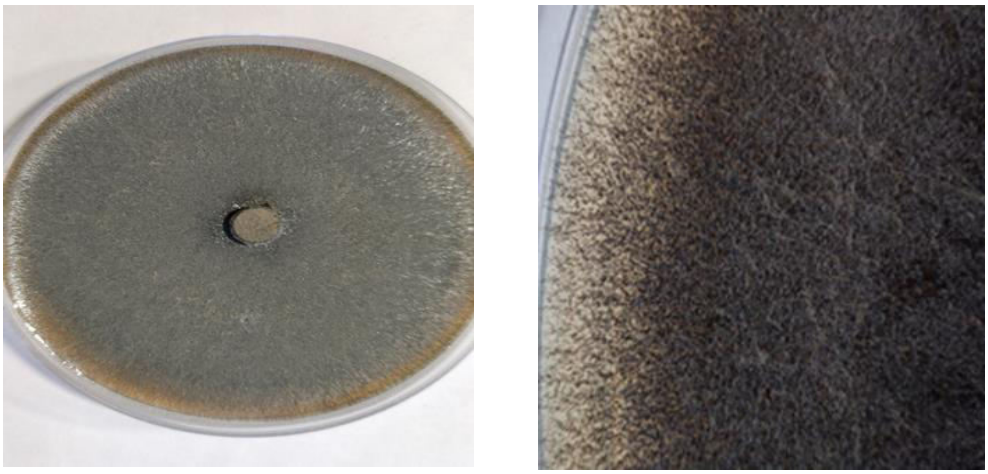
Results and Discussion

Morphological features of isolate gmax20 and gmaxr2

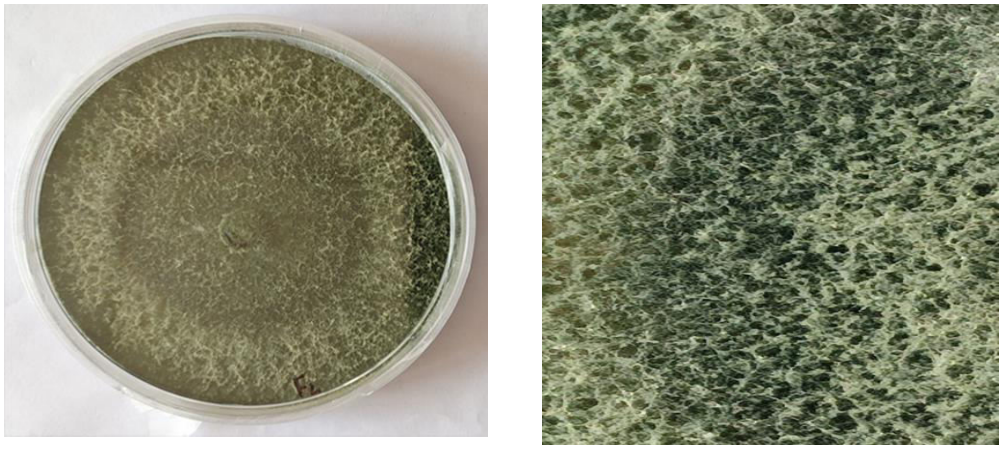
The fungal pathogen isolate Gmax20 was isolated using potato dextrose agar plate. Initially, the isolate Gmax20 produced white mycelia on the PDA plate which after maturation changes into brown and eventually to black colour (Fig.1).



A **B**

Fig 1: (A), Pure plate of *M. phaseolina* Gmax20 (B), zoom view of hyphae of *M. phaseolina* Gmax20

The antagonistic isolate Gmaxr2 was isolated by the serial dilution technique. The mycelium of isolate Gmaxr2 was initially white and after 2 to 3 days, turned into a whitish-green (Fig.2).



A **B**

Fig 2: (A), Pure plate of *T. rugulosum* Gmaxr2 (B), Zoom view of mycelia of *T. rugulosum* Gmaxr2

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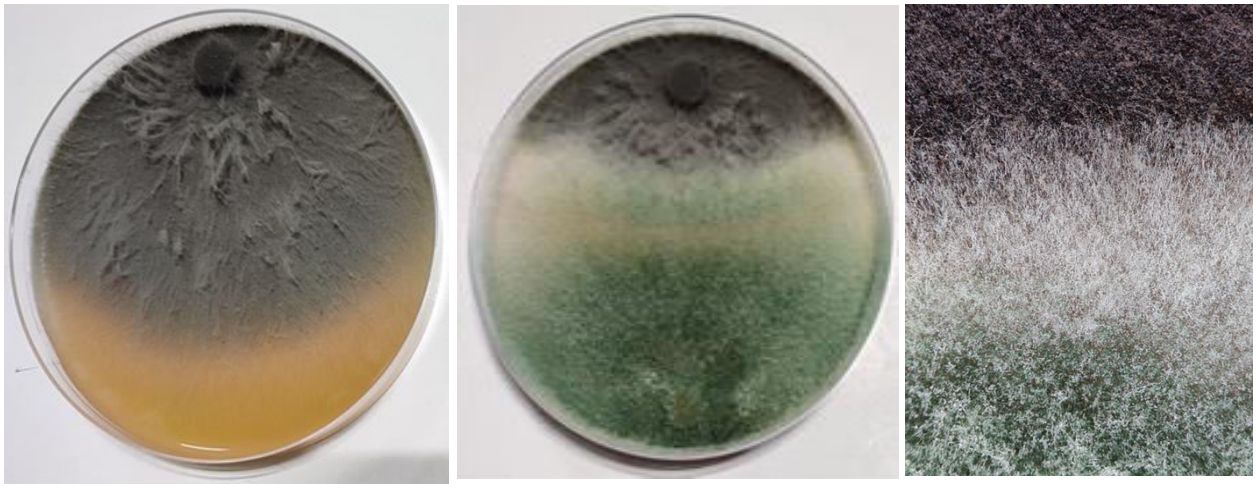
Molecular identification of isolate gmax20 and isolate gmaxr2

The pathogenic isolate (Gmax20) was confirmed as *Macrophomina phaseolina* Gmax20, whereas the antagonist isolate (Gmaxr2) was *Trichoderma rugulosum* Gmaxr2 through ITS region sequencing in molecular characterization. The amplified PCR product showed 100% similarity to previously submitted sequences in NCBI. The sequences of ITS were submitted and accession numbers OM004744 (isolate Gmax20) and OM638647 (isolate Gmaxr2) respectively were provided by the NCBI.

Molecular characterization is necessary for the accurate identification of fungal species because the morphological characteristics are misleading[17]. Moreover, to differentiate the species from each other, ITS region amplification and sequencing are highly trusted[16]. So, in the present study, molecular characterization of *Macrophomina phaseolina* Gmax20 and *Trichoderma rugulosum* Gmaxr2 was performed to gain accurate identification.

Efficacy of isolate gmaxr2 against pathogenic isolate gmax20 by dual plate method

The antagonistic activity of *Trichoderma rugulosum* Gmaxr2 against *Macrophomina phaseolina* Gmax20 using the dual plate method is shown in figure 3.



A **B** **C**

Fig.3: Antagonistic activity of *T. rugulosum* Gmaxr2 against *M. phaseolina* Gmax20 in dual plate method (A) Pure plate of *M. phaseolina* Gmax20, (B) *M. phaseolina* Gmax20 + *T. rugulosum* Gmaxr2, (D) zoom view of interaction zone of *M. phaseolina* Gmax20 + *T. rugulosum* Gmaxr2

The results depicted that *T. rugulosum* Gmaxr2 restricted the growth of *M. phaseolina* Gmax20 by 11.32%, 36.75%, and 50.52% on the first, second and third day respectively. It was also observed that *Trichoderma rugulosum* Gmaxr2 showed that maximum inhibition of *Macrophomina phaseolina* Gmax20 on the third day of the growth as compared to control (Fig. 4).

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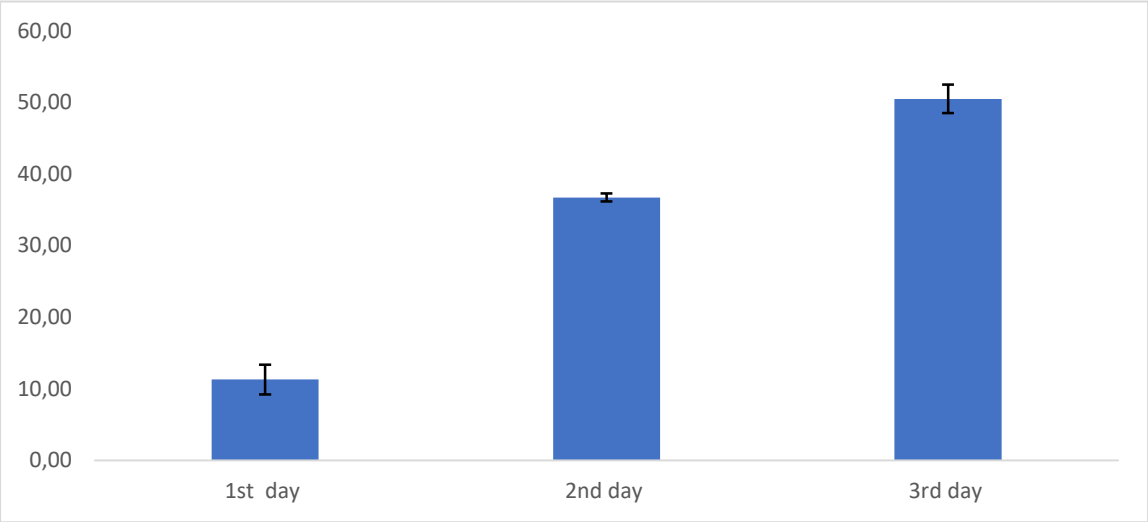

Fig 4. Inhibition % of *Macrophomina phaseolina* Gmax20 at 1st, 2nd, and 3rd day

The restricted growth of *Macrophomina phaseolina* Gmax20 in dual plate methods may be due to the secretion of growth inhibiting substances by *Trichoderma rugulosum* Gmaxr2. Deng et al. also observed that inhibited growth of pathogen could be due to the release of inhibitory substances, mycoparasitism, or competition for food and space[8]. Likewise, Gajera et al. reported that species of *Trichoderma* inhibited the hyphal growth of plant pathogens by antibiosis[11].

The finding of this study depicted that *Trichoderma rugulosum* Gmaxr2 showed remarkable potential in controlling the growth of *Macrophomina phaseolina* Gmax20, which is in accordance with Khan et al., Khalili et al., Hingole and Kendre[17,15,13].

Mycoparasitic activity

The struggle for nutrition and space is a basic cause of the mycoparasitic activity of *Trichoderma* species in the dual culture plate technique. In the present study, *Trichoderma rugulosum* Gmaxr2 showed mycoparasitic activity and completely reduced the hyphal growth of *Macrophomina phaseolina* Gmax20 on the 3rd day after antagonist started growing upon the pathogen (Fig. 3). The rapid growth of the hyphae of *Trichoderma rugulosum* Gmaxr2 occupies more nutrition and space in the Petri plate, which makes it more competitive against *Macrophomina phaseolina* Gmax20.

Compound light microscope observation

A study of compound light microscope showed the hyphal interaction of *Trichoderma rugulosum* Gmaxr2 and *Macrophomina phaseolina*. The results showed that within 3 days of incubation on PDA Petri plates, *M. phaseolina* Gmax20 established hyphal attachment to *T. rugulosum* Gmaxr2. In the present study, 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 (Fig. 5 C.)

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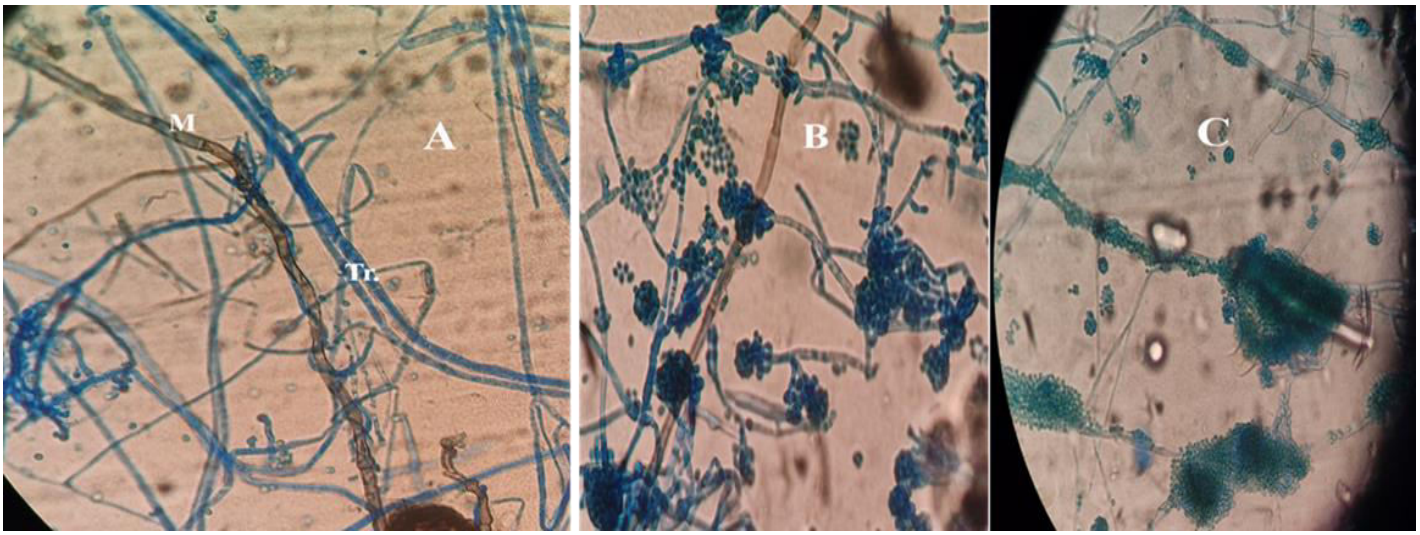
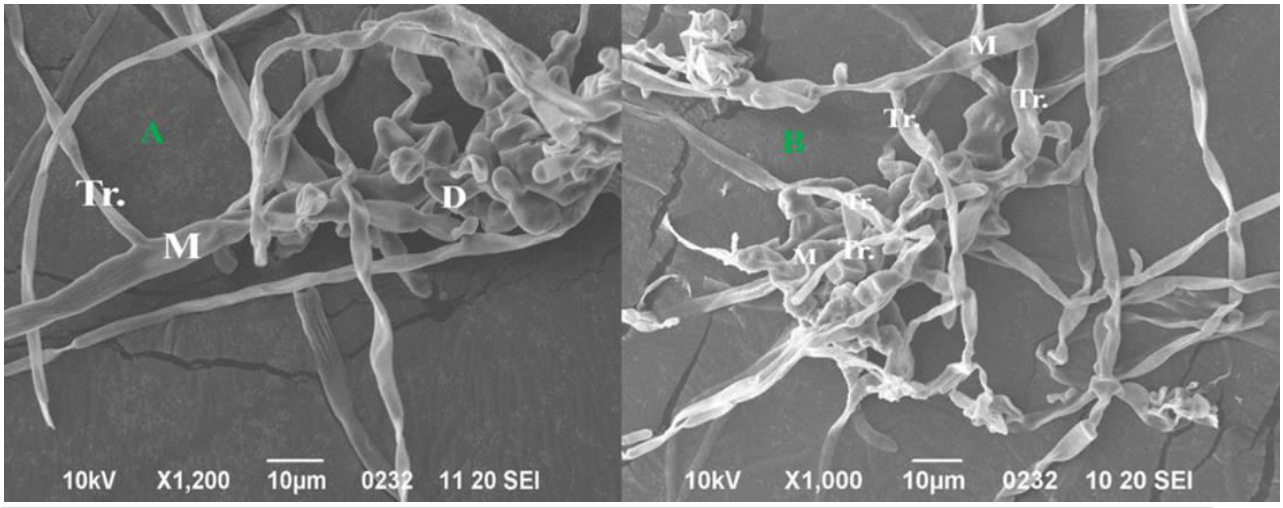


Fig.5: Images of a compound light microscope (40x) showing mycoparasitism of *T. rugulosum* Gmaxr2 on *M. phaseolina* Gmax20 (A-B) hyphal interaction between *T. rugulosum* Gmaxr2 and *M. phaseolina* Gmax20, and *T. rugulosum* Gmaxr2 (Tr.) hyphae attaching, penetrating, coiling, and deforming of *M. phaseolina* Gmax20(C), Spore concentration of *T. rugulosum* Gmaxr2 on the hyphae of *M. phaseolina* Gmax20.

The increased hyphal growth of *T. rugulosum* Gmaxr2 may form an appressoria-like structure on the hyphae of *M. phaseolina* Gmax20 that might be responsible for causing cytoplasmic coagulation. Moreover, the results also showed that *T. rugulosum* Gmaxr2 produced more branches around the hyphae of *M. phaseolina* Gmax20 and starts penetration and mycoparasitism behaviour by coiling and tightening the hyphae of the pathogen (Fig.5A). Furthermore, the antagonist *T. rugulosum* Gmaxr2 may destroys cell wall integrity using the cell fluid as a nutrient source which might leads to thinning, rupturing, deformation, and lysis in the hyphae of *M. phaseolina* Gmax20 (Fig. 6).

Scanning electron microscope

The mycoparasitic activity of *Trichoderma rugulosum* Gmaxr2 against *Macrophomina*



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phaseolina Gmax20 was clearly observed in the micrograph of SEM (Fig 6).

Fig.6: SEM images of Mycoparasitism of *T. rugulosum* Gmax20 on *M. phaseolina* Gmax20(A-B) hyphal interaction between *T. rugulosum* Gmaxr2 and *M. phaseolina* Gmax20, *T. rugulosum* Gmaxr2 (Tr.) hyphae attaching, penetrating, coiling, and deformatimg *(*D) *of M. phaseolina* Gmax20 (M) *mycelium*

In the present study, the SEM images depicted that the hyphae of parasitic fungi 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. Moreover, the micrograph of SEM also showed the deformation of the pathogenic hyphae after the penetration of antagonist hyphae.

Earlier, it has also been reported that *Trichoderma* species showed mycoparasitic activity by coiling and secreting hydrolytic enzymes for the lysis of host hyphae [23,14]. In addition, during mycoparasitic activity, *Trichoderma* species secrete extracellular enzymes such as proteolytic, 1,3-glucanolytic, and chitinase that degrade the cell wall of fungal pathogens[17].

In this study, the SEM images also showed the penetration as well as lysis of pathogenic fungi which may be attributed to secretion of certain chemical substances by *Trichoderma rugulosum* Gmaxr2 to arrest the growth of pathogenic fungi *Macrophomina phaseolina* Gmax20. El-Benawy et al. further observed that hydrolytic enzymes produced by *Trichoderma* spp. creates grooves and rupture the host hyphae which strongly prevent the production of sclerotia as well as arrest the growth of pathogens[9].

CONCLUSION

The present study concluded that *Trichoderma rugulosum* Gmaxr2 inhibited the growth of *Macrophomina phaseolina* Gmax20 under invitro condition. In this study, the decreased growth of *M. phaseolina* Gmax20 by 50.52 % compared to control conditions significantly indicated that the growth of fungus *Macrophomina phaseolina* Gmax20 by mycoparasitism under invitro condition. Further, the effectiveness of *Trichoderma rugulosum* Gmaxr2 against *Macrophomina phaseolina* Gmax20 was also evident from the compound microscope and SEM images. Overall, this study concluded that *Trichoderma rugulosum* Gmaxr2 could serve as an effective biological control against *Macrophomina phaseolina* Gmax20.

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