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Suppression of *Sclerotium rolfsii* and *Fusarium oxysporum* through Glomalin a Glycoprotein Produced by Arbuscular Mycorrhizal Fungi under *in vitro* Condition

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Authors' contributions

This work was carried out in collaboration between both authors. Both authors read and approved the final manuscript.

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ABSTRACT

For sustainable agriculture to succeed, especially in the production of organic food, the use of biofertilizers and biopesticides is essential. Arbuscular mycorrhizal symbiosis is important for improving plant development through increased nutrient intake, soil stabilization, and carbon sequestration through the creation of glomalin, a distinct and specialized protein. It was not determined whether glomalin affects soil-borne fungi infections. Therefore, using glomalin isolated from the soil used to raise maize plants inoculated with various arbuscular mycorrhizal fungi (AMF), the inhibition of soil-borne plant diseases *Sclerotium rolfsii* and *Fusarium oxysporum* was

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examined. The outcomes demonstrated that there was variation in the quantity of glomalin generated by various AMF species. Glomalin isolated from soil inoculated with *Glomus coronatum* suppressed *Sclerotium rolfsii* and *Fusarium oxysporum* more effectively in *in-vitro* tests than soil inoculated with *Glomus intraradices* and *Glomus mosseae*.

Keywords: Fusarium oxysporum; glomalin; Glomus coronatum; Glomus intraradices; Glomus mosseae; Sclerotium rolfsii.

1. INTRODUCTION

AM fungal symbiosis with roots of most plants is very crucial because of its ability to increase plant growth [1], protection of plants against plant pathogens [2,3], and drought stress [4,5]. "One of the compounds produced by AM fungi is a recalcitrant glycoprotein, glomalin [6] which enhances soil aggregation" [7,8]. "This molecule is a hydrophobic, iron-binding glycoprotein-aceous substance that contains the major fraction of organic matter and is important for maintaining the long existence of soil texture. It shares many similarities with other biomolecules, such as hydrophobins [9], transferrins [10], and humic substances" [11]. "Euphoric amounts of glomalin are due to an abundance of hyphae in soil (more than 100 m cm^{-3}) [12] and the slow decomposition rate of glomalin (7 to 42 years)" [13,14]. Extracted glomalin contains tightly bound iron, organic matter, amino acids, and carbohydrates.

"Glomalin extracted from sand-based pot cultures of various AM fungi is equivalent to glomalin extracted from soil according to protein banding on sodium dodecyl sulfate-polyacrylamide gel electrophoresis, ELISA, glycoprotein assays, C, N, and H concentrations and NMR spectra" [15-17]. "Glomalin has a crucial role in the stabilization of aggregates by sloughing off hyphae onto the surrounding organic matter, binding to clays, and providing a hydrophobic coating" [18]. "The immunoreactive concentration of glomalin is positively correlated with the percent water-stable soil aggregates in both agricultural and native soils in many of the experiments" [19,7,6,16,18]. "Glomalin is not exuded by AM hyphae but is instead contained within hyphal walls" [20]. "After AM hyphal decomposition glomalin residue is left in the soil" [21]. "Standing stocks of hyphae in soil are on the order of 5 to 90 g C m^{-2}" [22].

"Many mechanisms have been proposed to play a role in plant protection by AMF; mainly improving plant nutrition, competition for colonization sites, and activation of plant defense mechanisms" [23]. "The role of AM fungi in biocontrol and plant tolerance to disease was already proven" [24,25]. However, the role of glomalin in the suppression of *Sclerotium rolfsii* and *Fusarium oxysporum* has been worked out probably for the first time.

2. MATERIALS AND METHODS

The current study was done in the Department of Biological Sciences, CBSH, Govind Ballabh Pant University of Agriculture and Technology, Pantnagar. Such type of research work with glycoprotein, Glomalin in the suppression of fungal pathogens was a novel kind of work.

2.1 Production of AMF Inoculum

"AMF inoculum of *Glomus coronotum*, *G.mosseae*, and *G. intraradices* was produced separately for two cycles of 60 days each on sorghum in steam sterilized soil: sand (1:1) mixture. The seeds of sorghum were sterilized with 0.1% Clorox solution followed by four rinses with sterilized water. The infective propagules from these inoculums were measured" [26].

2.2 Production of Glomalin

"One hundred infective propagules each of *G. coronatum*, *G. mosseae,* and *G. intraradices* were used to inoculate maize (*Zea mays* L.) seeds variety Kohinoor Special in 500 ml pots containing steam sterilized soil: sand (1:1) mixture. Maize seeds were surface sterilized following the same method mentioned above. The AMF inoculum was provided in holes and one germinated maize seed was kept on to the inoculum and covered with the same pot soil. Plants were watered three times a week with autoclaved distilled water and Hoagland's solution was added weekly having ¼ dose of phosphorus. Plants without inoculation of any AMF served as control, however, they did receive microbial wash from one gram inoculum of AMF which was obtained by filtering 1g inoculum through Whatman filter paper no. 1". The experiment was conducted in a greenhouse with 18h of light (600µmol photons m^{-2} s^{-1}) and

$27^0C \pm 2^0C$ temperature until plant senesced (80 days) (Fig. 1). Each treatment (inoculated and uninoculated) was replicated four times. The same pots were used to replant the maize for another 80 days.

2.3 Extraction, Purification, Precipitation, and Dialysis of Protein

After harvesting the plants, soil from each replicate was mixed thoroughly for extraction of glomalin. Total glomalin was extracted in 8 ml of 50 mM citrate buffer pH 8.0 at 121C for 90 min autoclaving [16]. The supernatant was collected after centrifugation at 10,000xg for 15 min. Glomalin extraction was continued until no reddish-brown color was left in the supernatant. All supernatant was pooled in an Oakridge tube (50 ml). The extraction process was repeated twice from the same sample. After extraction, precipitation was carried out in 1 N HCl and reconstituted in 100mM sodium borate (pH 8.0). Dialysis was done with 10 mM sodium borate at pH 8.0. Glomalin samples were lyophilized and again reconstituted in distilled water. Reconstituted protein was stored at -20^0C.

2.4 Estimation of Glomalin

"Glomalin was estimated by modified Bradford dye binding assay [8] with BSA as the standard and concentration was extrapolated to mg/g soil".

2.5 Source of Organisms

Pure cultures of various fungi were obtained from the Department of Plant Pathology, College of Agriculture, G.B.P.U.A & amp; T., Pantnagar. The cultures were maintained throughout the experiments.

2.6 Preparation of Culture Media

Fungus pathogens were cultured on potato-dextrose-Agar (PDA) having the following composition: Potato 200 gm. Dextrose 20 gm, Agar 20 g, Distilled water 1000 ml having pH 7.0.The media was sterilized at 15 psi for 20 minutes at 121ºC, in an autoclave.

2.7 Preparation and Maintenance of Culture

The test fungi were transferred with a sterilized nichrome 100 p on the PDA, present in a Petri plate and incubated for 5 days at 25+2ºC.

2.8 *In-vitro* Evaluation and Estimation of Inhibition of *S. rolfsii* and *F. oxysporum*

"Potato dextrose agar (PDA) was used for the growth of *Sclerotium rolfsii* and *Fusarium oxysporum.* The poisoned food technique was used to see the impact of glomalin on *S .rolfsii* and *F. oxysporum"* [27]. In control, the extractant was taken from the control pot grown in the absence of AMF. Two concentrations; 0.4 and 0.8 mg/Petri plate of glomalin, were used from three species of *G. coronatum*, *G. intraradices,* and *G. mosseae*, separately. Discs of 5 mm diameter were taken from a full-grown Petri plate of either *S.rolfsii* or *F.oxysporum* and were placed over glomalin containing as well as control Petri plates. Each treatment was replicated 4 times. Growth inhibition was measured as the reduction in the radial growth of the pathogen with glomalin over control. Percent inhibition was calculated using the formula:

$$\frac{C-T}{T} \times 100$$

Where C and T are radial growth of the pathogen in control and treatment, respectively.

2.9 Statistical Analysis

The data were analyzed by one-way ANOVA.

3. RESULTS

3.1 Plant Growth and Production of Glomalin

Significant growth enhancement of maize was observed in either of the AMF-inoculated plants (data not shown). Maize (*Zea mays* L.) plants inoculated with *G. coronatum* showed significantly higher shoot and root dry weight followed by *G. intraradices* and *G. mosseae*.

The color of total glomalin from pot soil was dark brown in *G. coronatum,* yellow-brown in *G. mosseae,* and red-brown in *G. intraradices*. A negligible amount of glomalin was found in the control. A significantly higher amount of glomalin was recorded in the case of *G. coronatum* followed by *G. intraradices* and *G. mosseae* (Table 1).

3.2 Percent Inhibition of *S. rolfsii* and *F. oxysporum*

Percent inhibition of *S. rolfsii* and *F. oxysporum* was highest by glomalin extracted from pot

inoculated with *G. coronatum* (Figs. 2 & 7) followed by *G. intraradices* (Figs. 3 & 6) and *G. mosseae* (Figs. 4 & 5) (Table 2). The percent inhibition of pathogen was higher at higher concentrations of glomalin in Petri plates (Table 2). All the data was significantly different at p ≤0.05.

4. DISCUSSION

"There is accumulating evidence that AMF can reduce disease incidence and propagule number of several soilborne pathogens including *Aphanomyces*, *Fusarium*, *Phytophthora*, *Pythium*, and *Verticillium* species in the plant and mycorrhizosphere" [28-33]. "Although the mechanisms implicated are still not well characterized, direct and indirect interactions between AMF and pathogens have been put forward as a plausible hypothesis to explain the role of AMF in the biological control of root diseases" [34]. The present finding is additional information proving the role of glomalin in the suppression of soil-borne pathogens.

"In this experiment shoot and root growth of maize plants varied with different mycorrhizal species: *G.coronatum*, *G.mosseae* and *G.intraradices*. It indicates that different species of AMF show different effects on the same variety of plants. Glomalin production was found significantly higher in soils used to grow plants with *G. coronatum* followed by *G. intraradices* and *G. mosseae*. This could be the result of the performance of an AMF species to produce extraradical hyphae which is ultimately responsible for the release of glomalin in soil. Significant plant growth enhancement by *G .coronatum* reflects more functionality of extraradical hyphae and therefore, there is a possibility that the amount of extraradical hyphae is more in this case and so the amount of glomalin. A negligible amount of glomalin in the control pots assures the production of this glycoprotein from AMF only. This describes imploding of glomalin from different species of AMF as a variable" [35].

In addition to the shifting function of glomalin in standing stocks of soil in response to AMF communities, plant dynamics, inorganic resources (e.g., N, P, and atmospheric CO_2), and land use regimes, "a new putative focusing result was found, which provide a new possible and promising explanation to the involvement of AM fungi in plant protection against soil-borne pathogens, and point to their use as biological control agents" [35].

Table 1. Concentration of glomalin from different AMF pure culture

S. No.	AMF Species	Glomalin (mg/g soil)
1	*G.coronatum*	14.27
2	*G.mosseae*	10.2
3	*G.intraradicis*	10.64
4	Control	0.0
	LSD (p ≤0.05)	0.12

Fig. 1. Plant growth inoculated with AMF
Con- Control soil without AMF
C- Soil inoculated with G. Coronatum
I- Soil inoculated with G. intraradices
M- Soil inoculated with G. Mosseae

Table 2. Percent Inhibition of *S. rolfsii* and *F. oxysporum* at different concentrations of glomalin

		Percent Inhibition of *S. rolfsii*		Percent Inhibition of *F. oxysporum*	
S. No.	**AMF Species**	**% inhibition at 0.8 mg glomalin /petriplate**	**% inhibition at 0.4 mg glomalin /petriplate**	**% inhibition at 0.8 mg glomalin /petriplate**	**% inhibition at 0.4 mg glomalin /petriplate**
1	*G.coronatum*	69.03(56.19)	21.56(27.67)	69.47(56.46)	19.77(26.39)
2	*G.intraradices*	29.8(33.02)	12.3(20.51)	35.3(36.44)	11.63(19.93)
3	*G.mosseae*	20.1(26.61)	6.17(14.11)	27.83(31.82)	10.37(18.73)
4	Control	0.0(0.0)	0.0(0.0)	0.0(0.0)	0.0(0.0)
	LSD ($p \leq 0.05$)	3.60	3.63	2.31	2.15
	SEM	1.11	1.11	0.71	0.66

Angularly transformed values are given in parentheses
LSD is from transformed values

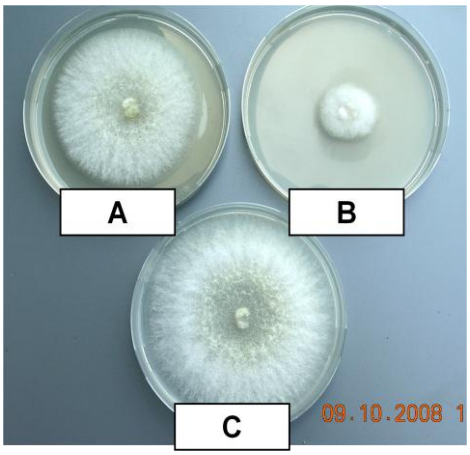

Fig. 2. Percent inhibition of *S. rolfsii* through glomalin from *G. coronatum*
A- Petri plate containing 0.4 mg glomalin
B- Petri plate containing 0.8 mg glomalin
C- Control without glomalin

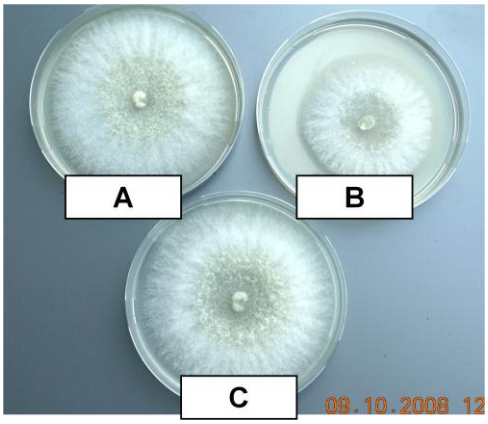

Fig. 3. Percent inhibition of *S. rolfsii* through glomalin from *G. intraradices*
A- Petri plate containing 0.4 mg glomalin
B- Petri plate containing 0.8 mg glomalin
C- Control without glomalin

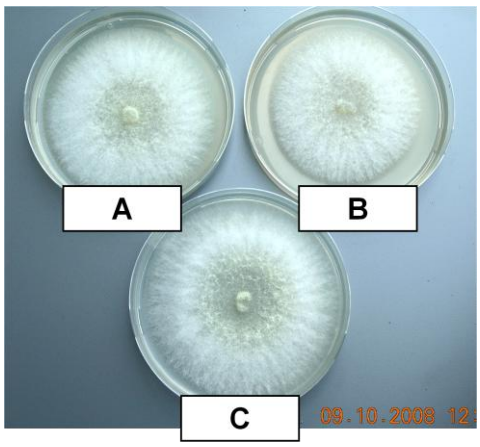

Fig. 4. Percent inhibition of *S. rolfsii* through glomalin from *G. Mosseae*
A- Petri plate containing 0.4 mg glomalin
B- Petri plate containing 0.8 mg glomalin
C- Control without glomalin

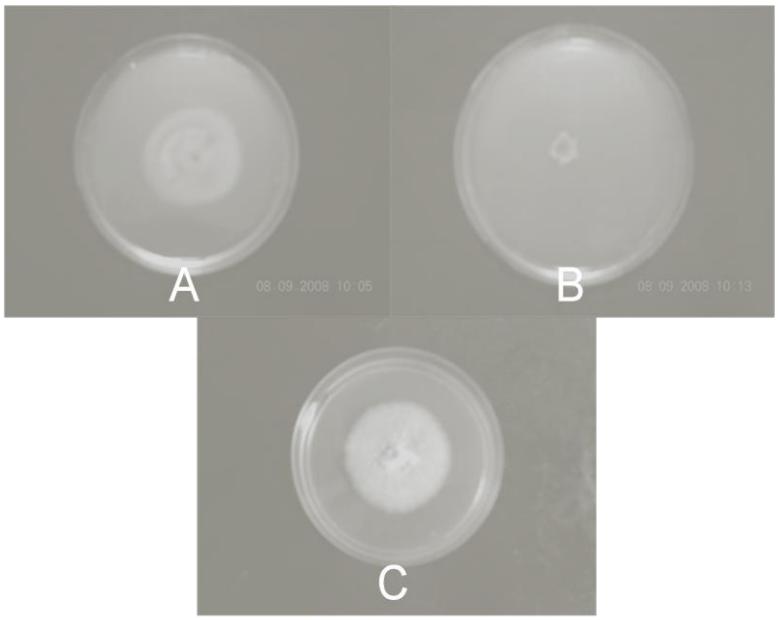

Fig. 5. Percent inhibition of *F. oxysporum* through glomalin from *G. Mosseae*
A- Petri plate containing 0.4 mg glomalin
B- Petri plate containing 0.8 mg glomalin
C- Control without glomalin

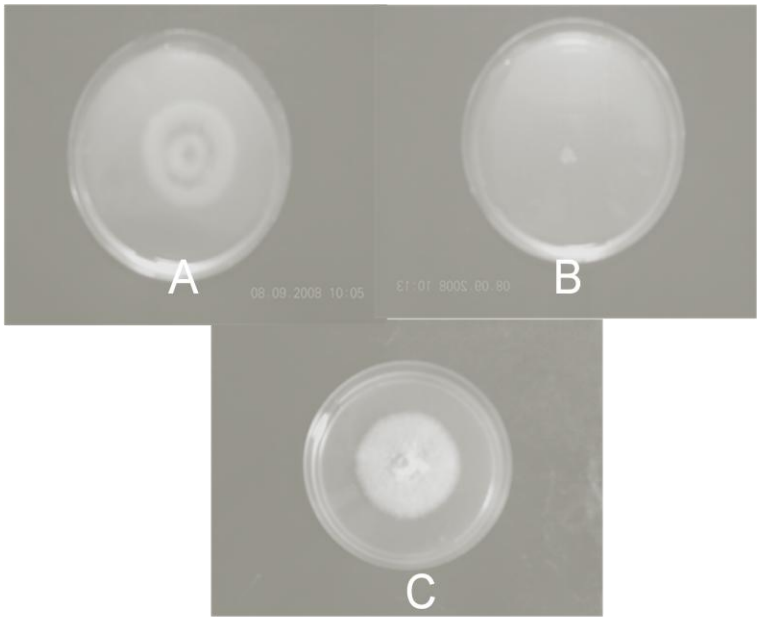

Fig. 6. Percent inhibition of *F. oxysporum* through glomalin from *G. intraradices*
A- Petri plate containing 0.4 mg glomalin
B- Petri plate containing 0.8 mg glomalin
C- Control without glomalin

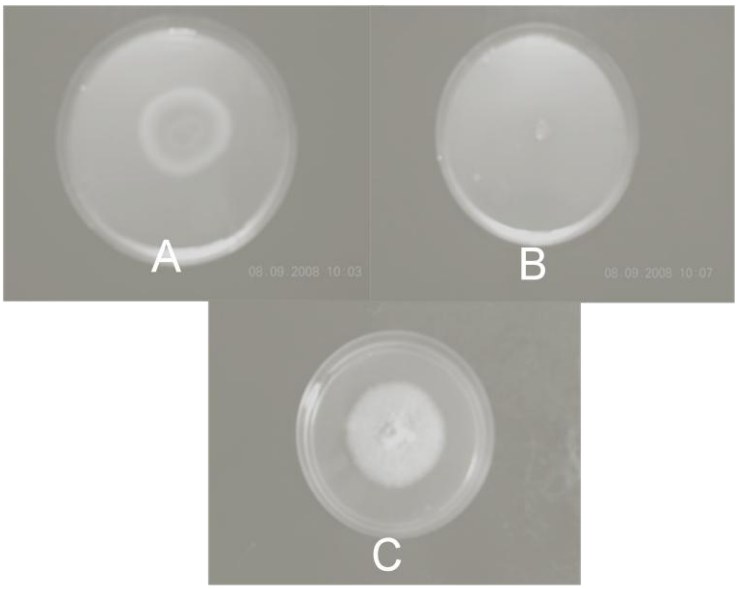

Fig. 7. Percent inhibition of *F. oxysporum* through glomalin from *G. Coronatum*
A- Petri plate containing 0.4 mg glomalin
B- Petri plate containing 0.8 mg glomalin
C- Control without glomalin

5. CONCLUSIONS

In vitro, studies showed that at different concentrations of glomalin percent, inhibition of *S.rolfsii* and *F.oxysporum* was significantly higher at higher concentrations of glomalin showing antifungal activity. The result indicates a direct antibiosis of glomalin in the suppression of pathogens under in vitro conditions. It might be possible that glomalin plays a very crucial role in the suppression of soil-borne pathogens in plants too.

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COMPETING INTERESTS

Authors have declared that no competing interests exist.

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