

Suppression of *Sclerotium rolfii* and *Fusarium oxysporum* through glomalin a glycoprotein produced by arbuscular mycorrhizal fungi under in vitro condition

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 rolfii* and *Fusarium oxysporum* was 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 rolfii* 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 rolfii*.

Introduction

AM fungal symbiosis with roots of most plants is very crucial because of its ability to increase plant growth (Smith & Read 1997), protection of plants against plant pathogens (Gange and West 1994; Borowicz 2001), and drought stress (Newsham et al. 1995; Gupta and Kumar 2000). “One of the compounds produced by AM fungi is a recalcitrant glycoprotein, glomalin (Wright et al. 1996) which enhances soil aggregation” (Wright & Upadhyaya 1998; Rillig et al. 2001). “This molecule is a hydrophobic, iron-binding glycoproteinaceous 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 (Wessels 1997), transferrins (Iyer &

Lonnerdal 1993), and humic substances” (Hayes & Clapp 2001). “Euphoric amounts of glomalin are due to an abundance of hyphae in soil (more than 100 m cm^{-3}) (Miller et al. 1995) and the slow decomposition rate of glomalin (7 to 42 years)” (Rillig et al. 2001; Steinberg & Rillig 2003). 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” (Wright & Upadhyaya 1996; Wright et al. 1998; Rillig et al. 2001). “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” (Wright & Upadhyaya 1999). “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” (Bird et al. 2002; Rillig et al., 2003; Wright & Anderson 2000; Wright & Upadhyaya 1998; Wright et al. 1999). “Glomalin is not exuded by AM hyphae but is instead contained within hyphal walls” (Driver et al. 2005). “After AM hyphal decomposition glomalin residue is left in the soil” (Treseder & Allen 2000). “Standing stocks of hyphae in soil are on the order of $5 \text{ to } 90 \text{ g C m}^{-2}$ ” (Zhu & Miller 2003).

“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” (Pozo et al. 2007). “The role of AM fungi in biocontrol and plant tolerance to disease was already proven” (Linderman 1994; 2000). However, the role of glomalin in the suppression of *Sclerotium rolfsii* and *Fusarium oxysporum* has been worked out probably for the first time.

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.

Production of AMF Inoculum

“AMF inoculum of *Glomus coronatum*, *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” (Sharma et al. 1996).

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”. (Sharma et. Al., 2017) The experiment was conducted in a greenhouse with 18h of light ($600\mu\text{mol photons m}^{-2} \text{s}^{-1}$) and $27^{\circ}\text{C} \pm 2^{\circ}\text{C}$ temperature until plant senesced (80 days) (Figure.1). Each treatment (inoculated and uninoculated) was replicated four times. The same pots were used to replant the maize for another 80 days.

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 8ml of 50mM citrate buffer pH 8.0 at 121°C for 90 min autoclaving (Wright et al. 1998). 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 (50ml). 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 °C.

Estimation of glomalin

“Glomalin was estimated by modified Bradford dye binding assay (Wright et al. 1996) with BSA as the standard and concentration was extrapolated to mg/g soil”.

Source of organisms

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

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 121°C, in an autoclave.

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±2 °C.

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*” (Finhold et al. 1951). In control, the extractant was taken from the control pot grown in the absence of AMF. Two concentrations; 0.4 and 0.8mg/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.

Statistical Analysis

The data were analyzed by one-way ANOVA.

Results

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).

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* (Figures. 2 & 7) followed by *G. intraradices* (Figures. 3 & 6) and *G. mosseae* (Figures. 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 $p \leq 0.05$.

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” (Hwang et al. 1992; Sharma et al. 1992; Liu 1995; Cordier et al. 1996; St-Arnaud et al. 1995; 1997). “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” (Azcón-Aguilar and Barea 1996). 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”. (Sharma et. Al., 2017)

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 CO₂), 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”. (Sharma et. Al., 2017)

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.

Acknowledgment

This research was conducted at CBSH, G B P U A & T Pantnagar from 2006-2011. I express my vehement sense of gratitude to the members of my advisory committee- Dr. A. K. Sharma, Professor, Department of Biological Sciences for their peerless advice, candid discussions, and valuable suggestions, and for providing Rhizosphere Biology Lab for my doctorate research throughout the course of my investigation.

References

- Azcón-Aguilar C, Barea JM (1996) Arbuscular mycorrhizas and biological control of soil-borne plant pathogens—an overview of the mechanisms involved. *Mycorrhiza* 6:457-464
- Bird SB, Herrick JE, Wander MM, Wright SF (2002) Spatial heterogeneity of aggregate stability and soil carbon in semi-arid rangeland. *Environ. Pollut* 116:445-455
- Borowicz VA (2001) Do arbuscular mycorrhizal fungi alter plant-pathogen relations? *Ecology* 82: 3057-3068
- Cordier C, Gianinazzi S, Gianinazzi-Pearson V (1996) Colonisation patterns of root tissues by *Phytophthora nicotianae* var. *parasitica* related to reduced disease in mycorrhizal tomato. *Plant Soil* 185:223-232
- Driver JD, Holben WE, Rillig MC (2005) Characterization of glomalin as a hyphal wall component of arbuscular mycorrhizal fungi. *Soil Biol. Biochem* 37:101-106
- Finhold RW (1951) Improved toximetric agar disk test for evaluation of wood preservative. *Anal. Chem* 23: 1038

- Gange AC, West HM (1994) Interactions between arbuscular mycorrhizal fungi and foliar-feeding insects in *Plantago lanceolata* L. *New Phytol* 128:79-87
- Gupta R, Kumar P (2000) Mycorrhizal plants in response to adverse environmental conditions. In; Mukerji KG, Chamola BP, Singh J (Eds), *Mycorrhizal Biology*, Kluwer Academic/Plenum Publishing, New York, pp 67-84
- Hayes MHB, Clapp CE (2001) Humic substances: Considerations of compositions, aspects of structure, and environmental influences. *Soil Sci* 166: 723-737
- Hwang SF, Chang KF, Chakravarty P (1992) Effects of vesicular-arbuscular mycorrhizal fungi on the development of *Verticillium* and *Fusarium* wilts of Alfalfa. *Plant Dis* 76:239-243
- Iyer S, Lonnerdal B (1993) Lactoferrin, lactoferrin receptors and iron metabolism. *Euro. J. Clin. Nutrit* 47:232-241
- Linderman RG (1994) Role of VAM fungi in biocontrol. In: Pflieger FL, Linderman RG (eds) *Mycorrhizae and plant health*. APS, St. Paul, Minn, pp 1-26
- Linderman RG (2000) Effects of mycorrhizas on plant tolerance to disease. In: Kapulnik Y, Douds DD (eds) *Arbuscular mycorrhizas: physiology of function*. Kluwer. Dordrecht, pp 345-365
- Liu RJ (1995) Effect of vesicular-arbuscular mycorrhizal fungi on *Verticillium* wilt of cotton. *Mycorrhiza* 5:293-297
- Miller MH, McGonigle TP, Addy HD (1995) Functional ecology of vesicular-arbuscular mycorrhizas as Influenced by phosphate fertilization and tillage in an agricultural ecosystem. *Crit. Rev. Biotechnol* 15:241-255
- Newsham KK, Fitter AH, Watkinson AR (1995) Arbuscular mycorrhiza protect an annual grass from root pathogenic fungi in the field. *J. Ecol* 83:991-1000
- Pozo MJ, Azcon-Aguilar C (2007) Unraveling mycorrhiza-induced resistance. *Curr Opin Plant Biol* 10:393-398
- Rillig MC, Ramsey PW, Morris S, Paul EA (2003) Glomalin, an arbuscular-mycorrhizal fungal soil protein, responds to land-use change. *Plant Soil* 253:293-299
- Rillig MC, Wright SF, Allen MF, Field CB (1999) Rise in carbon dioxide changes soil structure. *Nature* 400: 628
- Rillig MC, Wright SF, Nichols KA, Schmidt WF, Torn MS (2001) Large contribution of arbuscular mycorrhizal fungi to soil carbon pools in tropical forest soils. *Plant Soil* 233: 167-177
- Sharma AK, Johri BN, Gianinazzi S (1992) Vesicular-arbuscular mycorrhizae in relation to plant disease. *World J Microbiol Biotechnol* 8:550-563
- Sharma MP, Gaur A, Bhatia NP, Adholeya A (1996) Growth responses and dependence OF *Acacia nilitica* var. *cupriciformis* on the indigenous arbuscular mycorrhizal consortium of a marginal wasteland soil. *Mycorrhiza* 6: 441-446

- Smith SE, Read DJ (1997) Mycorrhizal symbiosis, 2nd edn. Academic, London, 605p.
- St-Arnaud M, Hamel C, Caron M, Fortin JA (1995) Inhibition of *Pythium ultimum* in roots and growth substrate of mycorrhizal *Tagetes patula* colonized with *Glomus intraradices*. Can. J. Plant Pathol 16:187-194
- St-Arnaud M, Hamel C, Vimard B, Caron M, Fortin JA (1997) Inhibition of *Fusarium oxysporum* f. sp. *dianthi* in the non-VAM species *Dianthus caryophyllus* by co-culture with *Tagetes patula* companion plants colonized by *Glomus intraradices*. Can. J. Bot 75:998-1005
- Steinberg PD, Rillig MC (2003) Differential decomposition of arbuscular mycorrhizal fungal hyphae and glomalin. Soil Biol. Biochem 35:191-194
- Treseder KK, Allen MF (2000) Mycorrhizal fungi have a potential role in soil carbon storage under elevated CO₂ and nitrogen deposition. New Phytol 147:189-200
- Wessels JGH (1997) Hydrophobins: Proteins that change the nature of the fungal surface. Adv. Microb. Physiol 38: 1-45
- Wright SF, Anderson RL (2000) Aggregate stability and glomalin in alternative crop rotations for the central Great Plains. Biol. Fert. Soils 31:249-253
- Wright SF, Franke-Snyder M, Morton JB, Upadhyaya A (1996) Time-course study and partial characterization of a protein on hyphae of arbuscular mycorrhizal fungi during active colonization of roots. Plant Soil 181: 193-203
- Wright SF, Upadhyaya A (1996) Extraction of an abundant and unusual protein from soil and comparison with hyphal protein of arbuscular mycorrhizal fungi. Soil Sci 161: 575-585
- Wright SF, Upadhyaya A (1998) A survey of soils for aggregate stability and glomalin, a glycoproteins produced by hyphae of arbuscular mycorrhizal fungi. Plant Soil 198: 97-107.
- Wright SF, Upadhyaya A (1999) Quantification of arbuscular mycorrhizal fungi activity by the glomalin concentration on hyphal traps. Mycorrhiza 8:283-285
- Zhu YG, Miller RM (2003) Carbon cycling by arbuscular mycorrhizal fungi in soil-plant systems. Trends Plant Sci 8:407-409
- Sharma S, Prasad R, Varma A, Sharma AK. Glycoprotein associated with *Funneliformis coronatum*, *Gigaspora margarita* and *Acaulospora scrobiculata* suppress the plant pathogens in vitro. Asian J Plant Pathol. 2017;11(4):199-202.

Table 1 Concentration of glomalin from Different AMF pure culture

S. No.	AMF Species	Glomalin (mg/g soil)
1	<i>G.coronatum</i>	14.27
2	<i>G.mosseae</i>	10.2
3	<i>G.intraradices</i>	10.64
4	Control	0.0
	LSD ($p \leq 0.05$)	0.12

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

		Percent Inhibition of <i>S. rolfisii</i>		Percent Inhibition of <i>F.oxysporum</i>	
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	<i>G.coronatum</i>	69.03(56.19)	21.56(27.67)	69.47(56.46)	19.77(26.39)
2	<i>G.intraradices</i>	29.8(33.02)	12.3(20.51)	35.3(36.44)	11.63(19.93)
3	<i>G.mosseae</i>	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

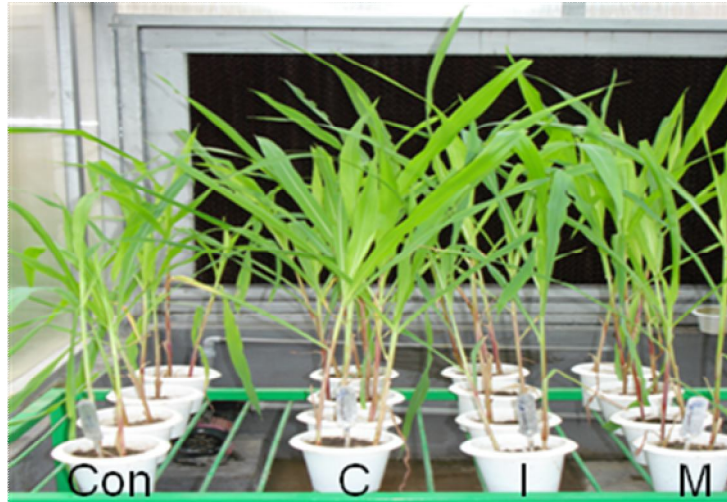


Figure.1 Plant growth inoculated with AMF

- Con- Control soil without AMF
- C- Soil inoculated with *G. Coronatum*
- I- Soil inoculated with *G. intraradices*
- M- Soil inoculated with *G. Mosseae*

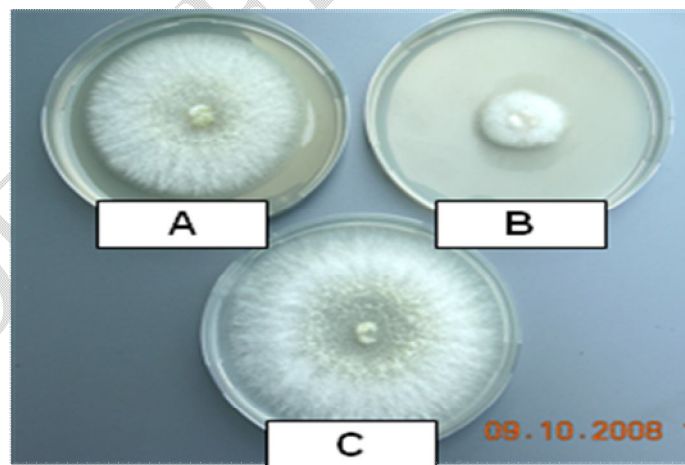


Figure.2 Percent Inhibition of *S. rolfsii* through glomalin from *G. coronatum*

- A- Petri plate containing 0.4 mg glomalin
- B- Petri plate containing 0.8 mg glomalin
- C- Control without glomalin

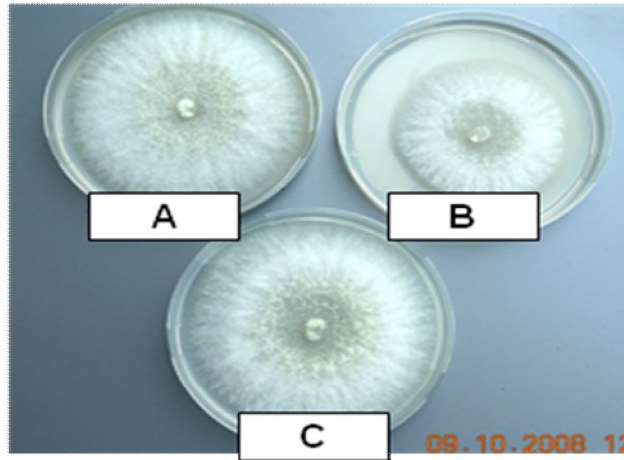


Figure. 3 Percent Inhibition of *S. rolfsii* through glomalin from *G. intraradices*

- A- Petri plate containing 0.4 mg glomalin
- B- Petri plate containing 0.8 mg glomalin
- C- Control without glomalin

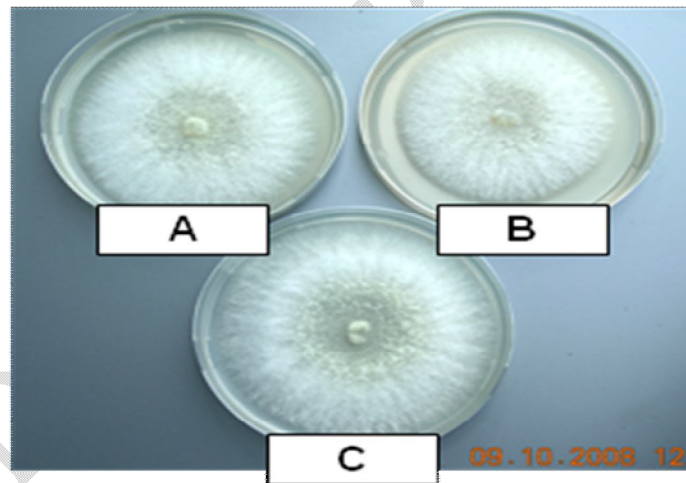


Figure. 4 Percent Inhibition of *S. rolfsii* through glomalin from *G. Mosseae*

- A- Petri plate containing 0.4 mg glomalin
- B- Petri plate containing 0.8 mg glomalin
- C- Control without glomalin

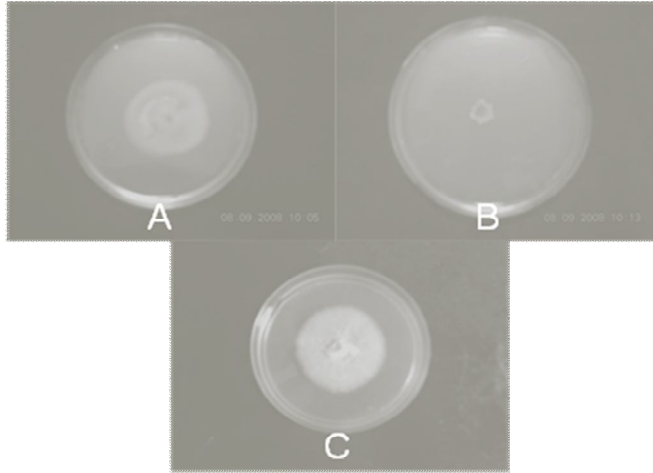


Figure.5 Percent Inhibition of *F.oxysporum* through glomalin from *G. Mosseae*

A- Petri plate containing 0.4 mg glomalin

B- Petri plate containing 0.8 mg glomalin

C- Control without glomalin

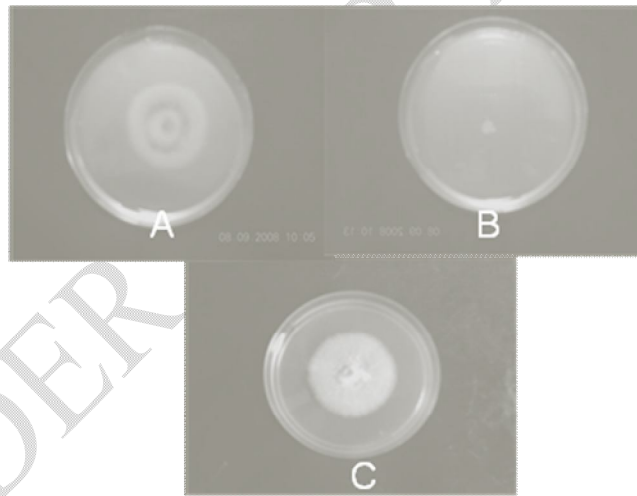


Figure.6 Percent Inhibition of *F.oxysporum* through glomalin from *G. intraradices*

A- Petri plate containing 0.4 mg glomalin

B- Petri plate containing 0.8 mg glomalin

C- Control without glomalin

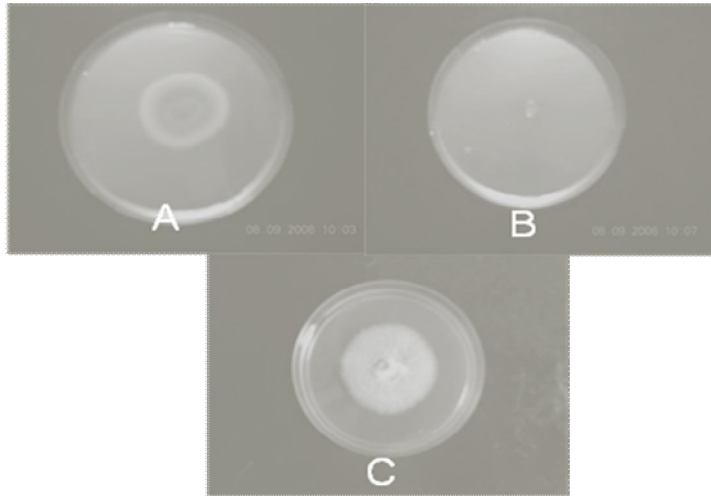


Figure.7 Percent Inhibition of *F.oxysporum* through glomalin from *G. Coronatum*

- A- Petri plate containing 0.4 mg glomalin
- B- Petri plate containing 0.8 mg glomalin
- C- Control without glomalin

UNDER PEER REVIEW