Original Research Article

In vitro suppression of soil borne plant pathogens by glomalin produced by arbuscular mycorrhizal fungi

Abstract Role of biofertilizers and biopesticides are being critical for sustainable agriculture especially in the production of green food. Arbuscular mycorrhizal symbiosis plays a significant role in plant growth improvement through higher nutrient uptake and in soil stabilization, carbon sequestration through the production of unique and specific protein: glomalin. The role of glomalin on soil borne fungal pathogens was not confirmed. Therefore, suppression of soil-borne plant pathogens *Sclerotium rolfsii* and *Fusarium oxysporum* was investigated using glomalin extracted from the soil used for raising maize plants inoculated with different arbuscular mycorrhizal fungi (AMF). The results showed that the amount of glomalin produced from different AMF species varied species to species. *In vitro* testing of suppression of *Sclerotium rolfsii* and *Fusarium oxysporum* by glomalin extracted from *Glomus coronatum* inoculated soil was higher followed by *Glomus intraradices* and *Glomus mosseae* inoculated soil.

Keywords: *Fusarium oxysporum*. Glomalin. *Glomus coronatum*. *Glomus intraradices*. *Glomus mosseae*. *Sclerotium rolfsii*. `

UNDER PEER REVIEW

Introduction

AM fungal symbiosis with roots of most of the land plants is very crucial because of its ability to increase plant growth (Smith & Read 1997), protection of plant 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 contains major fraction of organic matter and is important for maintaining 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 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 crucial role in stabilization of aggregates by sloughing off hyphae onto the surrounding organic matter, binding to clays and providing a hydrophobic coating (Wright & Upadhyaya 1999). Immunoreactive concentration of glomalin is positively correlated with 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 to 90 g C m^{-2} (Zhu &Miller 2003).

Many mechanisms have been proposed to play a role in plant protection by AMF; mainly improves plant nutrition, competition for colonization sites, and activation of plant defense mechanism (Pozo et al. 2007). Role of AM fungi in biocontrol and plant tolerance to disease was already proven (Linderman 1994; 2000). However, the role of glomalin in suppression of *Sclerotium rolfsii* and *Fusarium oxysporum* has been worked out probably for the first time.

Materials and methods

Production of Glomalin

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

UNDER PEER REVIEW

four rinsing with sterilized water. The infective propagules from these inoculums were measured (Sharma et al. 1996). One hundred infective propagules each of *G. coronatum*, *G. mosseae* and *G. intraradices* were used to inoculate maize (*Zea mays* L.) seeds variety------- 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 greenhouse with 18h of light (600µmol photons m^{-2} s^{-1}) and 27^0C ± 2^0C temperature until plant senesced (80 days). 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 121^0 C for 90 min autoclaving (Wright et al. 1998). Supernatant was collected after centrifugation at 10,000xg for 15 min. Glomalin extraction was continued until no reddish-brown color left in supernatant. All supernatant were pooled in an oakridge tube (50ml). 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 10mM sodium borate at pH 8.0. Glomalin samples were lyophilized and again reconstituted in distilled water. Reconstituted protein was stored at -20 ^{0}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.

UNDER PEER REVIEW

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.* Poisoned food technique was used to see the impact of glomalin on *S.rolfsii* and *F.oxysporum* (Finhold et al. 1951). In control, extractant was taken from control pot grown in 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 was taken from full grown Petri plate of either *S.rolfsii* or *F.oxysporum* and was placed over glomalin containing as well as control Petri plates. Each treatment was replicated 4 times. Growth inhibition was measured as reduction in the radial growth of pathogen with glomalin over control. Percent inhibition was calculated using formula:

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

Where C and T is radial growth of 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 were inoculated with *G. coronatum* showed significantly higher shoot and root dry weight followed by *G. intraradices* and *G. mosseae*.

Color of total glomalin from pot soil was dark brown in *G. coronatum,* yellow-brown in *G. mosseae* and red-brown in *G. intraradices*. Negligible amount of glomalin was found in control. Significantly higher amount of glomalin was recorded in case of *G. coronatum* followed by *G. intraradices* and *G. mosseae* (Table .1).

UNDER PEER REVIEW

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* followed by *G. intraradices* and *G. mosseae* (Table.2). The percent inhibition of pathogen was higher at higher concentration 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 biological control of root diseases (Azcón-Aguilar and Barea 1996). Present finding is additional information proving the role of glomalin in 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 shows different effects on same variety of plant. 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 hyphe and therefore, there is a possibility that the amount of extraradical hyphae is more in this case and so the amount of glomalin. Negligible amount of glomalin in the control pots assures the production of this glycoprotein from AMF only. This describes implode of glomalin from different species of AMF is variable. *In vitro* studies showed that at different concentrations of glomalin percent, inhibition of *S.rolfsii* and *F.oxysporum* was significantly higher at higher

UNDER PEER REVIEW

concentration of glomalin showing antifungal activity. The result indicates a direct antibiosis of glomalin in the suppression of pathogen under in vitro condition

In addition of 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.

UNDER PEER REVIEW

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UNDER PEER REVIEW

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UNDER PEER REVIEW

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

Table 2 Percent Inhibition of *S. rolfsii* and *F.oxysporum* at different concentration 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 ≤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 parenthesis.

LSD is from transformed values

UNDER PEER REVIEW