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RESEARCH ARTICLE

Impact of *Spodoptera litura* Attack on Chlorophyll and Biomass Content of *Vigna mungo* Colonized with Arbuscular Mycorrhizal Fungi and *Rhizobium*

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ABSTRACT

The first and foremost response of plants to any external stimuli, including herbivorous insect attack is the generation of reactive oxygen species, which majorly occurs in the chloroplast followed by mitochondria, peroxisomes, cell membrane, and cell wall. Under these circumstances, the photosynthetic efficiency of the plant system dramatically influences the plant biomass and yield. Although arbuscular mycorrhizal fungi (AMF), *Glomus intraradices* and *Rhizobium* improve plant growth through nutritional modes, their impact on protecting chloroplast from herbivore-induced damage is not yet explored. In this regard, experiments were carried out to examine the changes in chlorophyll composition (Chl a & b), and biomass production of *Rhizobium* brand AMF inoculated plants infested with *Spodoptera litura*. Among various treatments, AMF and *Rhizobium* inoculated plants showed higher chlorophyll a and b than un-inoculated control. Upon the herbivore attack, there was a drastic reduction in the chlorophyll content (a & b) in all the treatments. However, the reduction in chlorophyll content upon *S. litura* attack was lesser in plants inoculated either with AMF or in the combination of AMF and *Rhizobium.* Similarly, these microbial inoculants protected the plants from *S. litura* damage by sustaining the biomass productivity. These results highlight the synergistic effects of AMF and *Rhizobium* in protecting the black gram plants from herbivore-induced damage.

Keywords: Arbuscular mycorrhizal fungi*, Glomus intraradices, Rhizobium, Spodoptera litura,* chlorophyll*,* plant biomass

INTRODUCTION

Vigna mungo, a widely cultivated leguminous crop in India, forms a symbiotic association with *Rhizobium* and arbuscular mycorrhizal fungi (AMF). *Rhizobium* is a symbiotic diazotrophic bacterium that fulfills the nitrogen requirement of the plant by fixing atmospheric nitrogen. AMF increases plant growth by facilitating the absorption of immobile nutrients (phosphate, ammonium & micronutrients) and moisture through its extensive external hyphal network. These two beneficial microbes improve plant growth by increasing nutrient availability and modulating the physiological characteristics of the plants (Laouane *et al.,* 2019). Improved growth in mycorrhiza associated plants due to higher photosynthetic activity was reported (Kumar *et al.*, 2019). Earlier reports indicate that there is an increase in leaf area and chlorophyll content of mycorrhizae treated plants due to higher phosphorus availability (Dietz and Foyer, 1986; Xu *et al.*, 2018). Non-mycorrhizal plants contained lesser chlorophyll than those of mycorrhizal plants (Giri *et al.*, 2003). *Glomus etunicatum* (AMF) inoculated pistachio (*Pistacia vera* L.) seedlings recorded higher chlorophyll over non-mycorrhizal pistachio plants (Abbaspour *et al.*, 2012). Mycorrhizal plants had significantly higher chlorophyll, dry, and fresh biomass than plants are grown in sterilized soil (Zare-Maivan *et al.*, 2017).

Spodoptera litura is a polyphagous leaf-feeding insect responsible for a significant reduction in yield of black gram (Sreelakshmi *et al.*, 2019). Herbivorous insects induce biochemical and physiological changes in the host plants, affecting critical vital processes such as photosynthesis (Goławska *et al.*, 2010). It occurs in plants exposed to stress due to photo-oxidation of chlorophyll by the toxic oxygen species. In response to it, some plants develop photo-protective mechanisms to counteract the ill effects of toxic oxygen species through enzymatic and non-enzymatic modes. The decrease in chlorophyll content in response to a variety of stresses including insect feeding (Goławska *et al.*, 2010; Heng-Moss *et al.*, 2003; Ni *et al.*, 2002; Ni

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et al., 2001; Santamaría *et al.*, 2018) has been reported earlier. Thus, chlorophyll content could be an indicator of investigating plants' resistance to various stresses.

The plethora of earlier reports indicates that AMF improves plant chlorophyll and biomass production under abiotic stress conditions (Bagheri *et al.*, 2019; Goławska *et al.*, 2010; Huang *et al.*, 2014). Studies are imperative to address the effect of *S. litura* attack on the physiological properties of the plant *viz*., chlorophyll, and biomass productivity. Thus, studies were undertaken to investigate the effect of AMF and *Rhizobium* in protecting plants chlorophyll from biotic stress (*Spodoptera litura*).

MATERIAL AND METHODS

Microbial cultures

Glomus intraradices spores were obtained from the Department of Agricultural Microbiology, Tamil Nadu Agricultural University, Coimbatore. AM fungal spores were mass multiplied in sand using maize and used as a source of inoculum. *Rhizobium* BMBS strain was obtained from the Department of Agricultural Microbiology, Tamil Nadu Agricultural University, Coimbatore. It was multiplied in yeast extract mannitol (YEM) broth by incubating at 28 ˚C for 2 days in an Orbital shaker with 150 rpm. Then, the broth was centrifuged at 10000 rpm for 10 min under the refrigerated condition, and the cell pellet was collected. The cell pellet was then suspended in 1 mL of sterile distilled water and used as a source of inoculum.

Plant growth and microbial inoculation

Vigna mungo (CO 6) seeds used for this study were obtained from the Department of Pulses, Tamil Nadu Agricultural University, Coimbatore. Surface sterilized seeds (10 % sodium hypochlorite for 5 min followed by 70 % ethanol for 3 min. and then with sterile water 3- 4 times) inoculated with *Glomus intraradices* and *Rhizobium* were dibbled in 2 kg capacity pot containing a mixture of sterile red soil and sand at the ratio of 2:1. After seed germination, all the pots were irrigated alternatively with 100 mL Hoagland nutrient solution and 100 mL tap water at two days interval.

Insect treatment

Spodoptera litura egg mass was purchased from the National Bureau of Agricultural Insect Resources, Bengaluru. Insect eggs were kept in a plastic container with castor leaves until they reach the stage of third instar larvae and used as a source of insect material. After 40 days of sowing, plants were subjected to insect damage by releasing three number of third instar *Spodoptera litura* larvae. This investigation was carried out with eight treatments and three replications following a completely randomized design. The treatments include (C) without microbial inoculants and *S. litura*; (S) *S. litura*; (R) *Rhizobium*; (R*S) *Rhizobium* and *S. litura*; (G) *Glomus intraradices*; (G*S) G*. intraradices* and *S. litura*; (G*R) *G. intraradices* and *Rhizobium*; and (G*R*S) *G. intraradices*, *Rhizobium* and *S. litura*. The total number of 24 pots with three replications per treatment and one-pot per replication was maintained. Plants were uprooted after 12, 24, 48 and 96 h of *S. litura* treatment and used for assessing the chlorophyll content, plant biomass, mycorrhizal colonization, and root nodulation

Leaf chlorophyll

Chlorophyll was estimated based on the procedure of Arnon (1949) for which about 100 mg of fresh leaf tissue was macerated in 5 mL of 80 % acetone and then centrifuged for 10 min at 3000 rpm under refrigerated condition. This step was repeated until all the chlorophyll was extracted. Then, the extracts were pooled and the volume was made up to 10 ml with 80 % acetone. The intensity of green color was measured at 480 and 510 nm in a digital spectrophotometer (Spectramax® i3X) for chlorophyll a and chlorophyll b, respectively. Acetone (80 %) was used as a blank. The chlorophyll content was denoted as mg g^{-1} fresh leaf material.

Total biomass

Plants were harvested after 12, 24, 48, and 96 h of insect release and oven-dried at 70 ˚C for 4 h. The dry weight of shoot and root system was recorded and expressed as g $plant^{-1}$.

Nodulation and AM fungi colonization

Mycorrhizal colonization and nodulation were assessed at the end of the experiment. Plants were uprooted and washed gently with running tap water. First, nodules were counted and expressed as a number of nodules $plant^{-1}$. For mycorrhizal colonization, the roots were cut into small bits, treated with 10 % KOH solution, and autoclaved. Then, the root bits were treated with 2% HCl solution after decanting the alkali. After acid treatment, the roots were stained with 0.008% trypan blue for 24 h and then observed under a low power compound microscope. The mycorrhizal colonization was expressed in percentage (Phillips and Hayman, 1970).

Statistical analysis

Statistical analysis was done using the software, SPSS (Version 16.0), and Microsoft Excel. Values of mycorrhizal colonization and nodule number were transformed into arcsine (x/100) and log (x+1), respectively. One way analysis of variance (One way ANOVA) was done for the data concerned with

mycorrhizal colonization, and nodulation efficiency and the results were stated as mean with standard error (mean ± SE). Duncan's multiple range test (DMRT) was performed at $P < 0.05$ to compare the mean values. Three way analysis of variance (three-way ANOVA) was done to study the interaction effects of *G. intraradices*, *Rhizobium,* and the timing of insect release on the changes in chlorophyll a, b and total biomass production.

RESULTS AND DISCUSSION

The chlorophyll and biomass content of 40 days old black gram plants were measured at four different time intervals (12, 24, 48 and 96 h) after insect release in eight different treatments. As expected, the chlorophyll content was higher in inoculated plants over un-inoculated control. However, *S. litura* infestation reduced chlorophyll content and plant biomass production in all the treatments. However, the reduction in chlorophyll and biomass content was lesser when *S. litura* attacked AMF and *Rhizobium* inoculated plants.

Changes in chlorophyll content of AMF and Rhizobium inoculated black gram infested with S. litura

The concentration of chlorophyll-a of plants inoculated with AMF and *Rhizobium* (1.30 mg g^{-1} FW after 12 h) was almost doubled the concentration of control plants (0.63 mg g^{-1} FW after 12 h) in all the time intervals. The chlorophyll content increased due to *Rhizobium*, AMF, and dual inoculation with AMF and *Rhizobium* in all time intervals (Figure 1). The chlorophyll a content of plants did not reduce significantly when samples were analyzed after 12 h of herbivory ($P = 0.001$). However, the chlorophyll a content of plants of all the treatments reduced significantly from 24 of insect release to 96 h (Figure 1 & Table 1).

Table 1. Effect of Glomus intraradices, *Rhizobium* and *Spodoptera litura* on modulating chlorophyll a and b content of blackgram

Treatments	Chl a			Chl b	
	Df	*F*	*P*	*F*	*P*
G	1	1636.0	0.001	1025.0	0.001
R	1	245.87	0.001	181.24	0.001
S	1	259.73	0.001	38.033	0.001
T	3	57.190	0.001	2.904	0.041
G*R	1	82.380	0.001	17.395	0.001
G*S	1	4.0290	0.049	2.886	0.094
G*T	3	5.810	0.001	0.853	0.470
R*S	1	0.001	0.978	12.409	0.001
R*T	3	2.220	0.094	3.262	0.027
S*T	3	33.420	0.001	4.741	0.005
G*R*S	1	0.554	0.460	11.485	0.001
G*R*T	3	2.084	0.111	0.093	0.964
G*S*T	3	2.666	0.055	6.869	0.001
R*S*T	3	0.231	0.875	0.839	0.477
G*R*S*T	3	0.001	0.952	3.966	0.012
Error					64

The *P*- values indicate statistical significance ($p < .05$). C, Control; G, *G. intraradices*; R, *Rhizobium*; S, *S. litura*; T, Time.

In the absence of herbivory, both AMF and *Rhizobium* colonized plants recorded a higher concentration of chlorophyll a of 1.33 mg g^{-1} FW (24 h); while uninoculated control plants recorded minimum value of 0.30 mg g^{-1} FW after 96 h of insect attack (Table 1).

Similar to chlorophyll a, chlorophyll b content was higher in plants inoculated with both AMF and *Rhizobium* (1.34 mg g^{-1} FW). Uninoculated control plants exposed to *S. litura* recorded a minimum value of 01.6 mg g^{-1} FW after 96 h of herbivory (Figure 2). Even though microbial inoculants significantly improved the chlorophyll b content as that of chlorophyll a, herbivory treatment on uninoculated control, single inoculation with AMF and *Rhizobium* did not alter the chlorophyll content significantly ($P = 0.001$; Table 1). These plants registered almost similar concentrations of chlorophyll b in both insect attacked and normal plants till 48 h. A significant reduction in chlorophyll b content due to herbivory was observed only after 96 h of insect release in these treatments. However, *S. litura* attack on AMF and *Rhizobium* co-inoculated plants significantly reduced the chlorophyll content after 12 h of insect release itself. Compared to chlorophyll b, chlorophyll a was significantly influenced by various microbial treatments (Table 1 & Figure 2). However, AMF and *Rhizobium* inoculated plants showed a similar trend for chlorophyll a and b both in the presence and absence of insects. Similar to our study, Porra (2002) used leaf chlorophyll content as an indicator of plant health. The chlorophyll a: b ratio also specifies the

developmental state of photosynthetic apparatus in plants. Many reports indicated chlorophyll content and photosynthetic activity of plant tissue as primary physiological indicators of interactions between plants and herbivores (Goławska *et al.*, 2010; Golan *et al.*, 2015; Mohammed *et al.*, 2019).

Table 2. Effect of *Glomus intraradices*, *Rhizobium* and *Spodoptera litura* on plant biomass production of blackgram

Treatments	Plant biomass		
	Df	*F*	*P*
G	1	1386.0	0.001
R	1	450.049	0.001
S	1	123.740	0.001
T	3	17.494	0.001
G*R	1	294.042	0.001
G*S	1	0.012	0.914
G*T	3	1.354	0.265
R*S	1	14.411	0.001
R*T	3	0.928	0.432
S*T	3	27.973	0.001
G*R*S	1	0.277	0.601
G*R*T	3	0.847	0.473
G*S*T	3	0.088	0.967
R*S*T	3	1.508	0.221
G*R*S*T	3	4.803	0.004
Error			64

The *P*- values indicate statistical significance ($p < .05$). C, Control; G, *G. intraradices*; R, *Rhizobium*; S, *S. litura*; T, Time.

The chlorophyll content of *Bougainvillea* was higher in control plants than mealybugs infected plants, indicating that insect attack severely compromised the photosynthetic activity of infected plants (Abbate *et al.*, 2018). Losses in chlorophyll content of leaves of *Vitis vinifera* L have been reported in response to feeding by frosted scale, *Parthenolecanium pruinosum* (Simbiken *et al.*, 2015). Many reports have revealed that herbivore feeding affected the chlorophyll content of plants (Ramamurthy *et al.*, 1997; Heng-Moss *et al.*, 2003; Ni *et al.*, 2002; Mohammed *et al.*, 2019). AM fungal colonization significantly increased the leaf chlorophyll content (Formenti and Rasmann, 2019).

Table 3. Effect of *Glomus intraradices* infection potential in blackgram

Treatments	Mycorrhizal infection (%)							
	12h		24h		48h		96h	
C	0.00		0.00		0.00		0.00	
S	0.00		0.00		0.00		0.00	
R	0.00		0.00		0.00		0.00	
R*S	0.00		0.00		0.00		0.00	
G	0.99	0.06[b]	1.15	0.05[a]	1.15	0.047[a]	1.12	0.07[a]
G*S	1.12	0.07[a]	0.96	0.04[b]	1.12	0.07[a]	1.11	0.07[a]
G*R	1.08	0.11[b]	1.15	0.04[a]	1.12	0.07[a]	1..20	0.05[a]
F*R*S	1.07	0.04[b]	1.08	0.09[a]	1.07	0.03[a]	1.15	0.04[a]
Df								7
F		116.724		208.048		191.122		193.157
P		0.000		0.000		0.000		0.000

Data in the table are expressed as mean ± SE. Mean values followed by the same alphabet do not differ significantly at $P \leq 0.05$ by DMRT. C, Control; G, *G. intraradices*; R, *Rhizobium*; S, *S. litura*.

Changes in plant biomass production of AMF and Rhizobium inoculated blackgram infested with S. litura

In this study, plants inoculated with AMF produced maximum biomass indicating the importance of AMF in improving plant growth and development. There was no change in biomass production in all the treatments due to herbivory when the biomass was analyzed after 12 h of insect release (Figure 3). In all time intervals, AMF and *Rhizobium* inoculated plants registered more biomass. AMF + *Rhizobium* inoculated plants showed maximum biomass productivity (3.49 g plant^{-1}); while uninoculated control plants recorded a minimum of 0.59 g plant^{-1}. In general, biomass production reduced only after 24 h of insect release (Table 2). Overall, biomass production was higher due to AMF and *Rhizobium* either singly or in combination irrespective of the insect attack. Similarly, Kempel *et al.* (2010)

Table 4. Effect of *Rhizobium* inoculation on root nodulation potential in blackgram

Treatments	Nodules (No. of nodules plant^{-1})			
	12h	24h	48h	96h
C	0.00	0.00	0.00	0.00
S	0.00	0.00	0.00	0.00
R	1.17 0.05^{b}	1.19 0.06^{b}	1.21 0.04^{c}	1.24 0.03^{c}
R*S	1.19 0.10^{b}	1.29 0.06^{b}	1.22 0.05^{c}	1.31 0.01^{bc}
G	0.00	0.00	0.00	0.00
G*S	0.00	0.00	0.00	0.00
G*R	1.38 0.03^{a}	1.39 0.02^{a}	1.34 0.05^{b}	1.36 0.03^{b}
F*R*S	1.43 0.01^{a}	1.42 0.03)^{a}	1.38 0.04^{a}	1.43 0.03^{a}
Df				7
F	307.370	434.029	464.664	1089.00
P	0.000	0.00	0.000	0.000

Data in the table are expressed as mean ± SE. Mean values followed by the same alphabet do not differ significantly at $P \leq 0.05$ by DMRT. C, Control; G, *G. intraradices*; R, *Rhizobium*; S, *S. litura*.

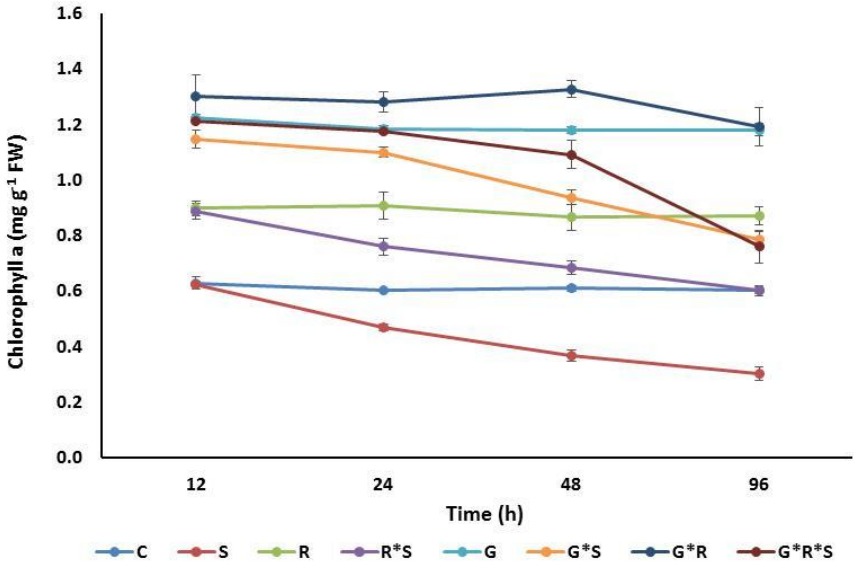

Figure 1. Effect of *Glomus intraradices*, *Rhizobium* and *Spodoptera litura* on modulating chlorophyll a content of blackgram at different time intervals (12, 24, 48 & 96 h). C, Control; G, G. *intraradices*; R, *Rhizobium*; S, S. *litura*. Data in the figure are expressed as mean ± SE. Mean values followed by the same letter do not differ significantly at P≤ 0.05 by DMRT.

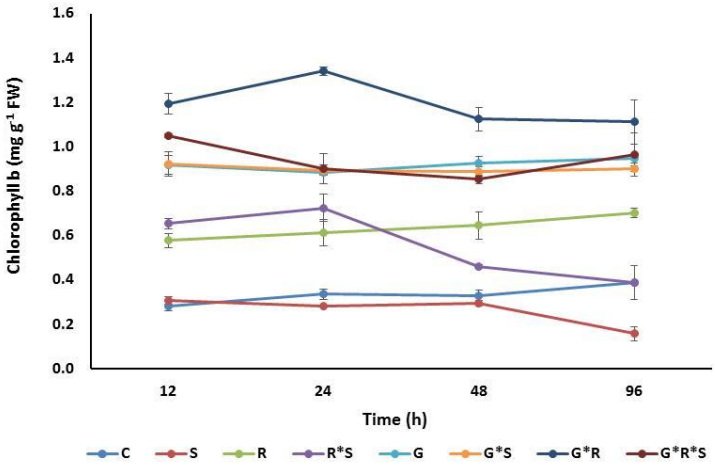

Figure 2. Effect of *Glomus intraradices*, *Rhizobium* and *Spodoptera litura* on modulating chlorophyll b content of blackgram at different time intervals (12, 24, 48 & 96 h). C, Control; G, G. *intraradices*; R, *Rhizobium*; S, S. *litura*. Data in the figure are expressed as mean ± SE. Mean values followed by the same letter do not differ significantly at P≤ 0.05 by DMRT.

reported an increase in above-ground plant growth of AMF colonized *Poa pratensis* in the absence of herbivory (*Spodoptera littoralis*). Similar to our study, Formenti and Rasmann, (2019) reported higher biomass in *Solanum lycopersicum* due to arbuscular mycorrhizal fungal colonization.

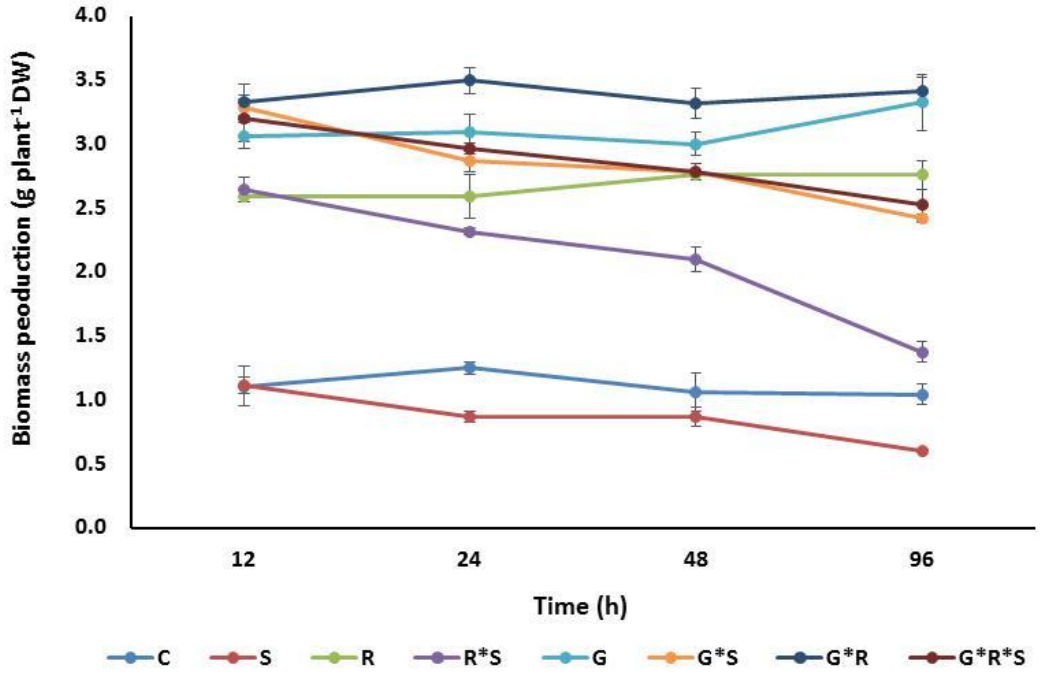

Figure 3. Effect of *Glomus intraradices*, *Rhizobium* and *Spodoptera litura* on modulating plant biomass production of blackgram at different time intervals (12, 24, 48 & 96 h). C, Control; G, G. *intraradices*; R, *Rhizobium*; S, S. *litura*. Data in the figure are expressed as mean ± SE. Mean values followed by the same letter do not differ significantly at P≤ 0.05 by DMRT.

Nodulation and AMF colonization ability of blackgram

Blackgram plants co-inoculated with AMF and *Rhizobium* produced more nodules per plant over plants treated with *Rhizobium* alone (Table 3). Plants treated with AMF and AMF + *Rhizobium* recorded maximum AMF colonization (Table 4). Thus, the results of the current study indicate that inoculation of blackgram plants microbial symbionts like AMF and *Rhizobium* could help in reducing the loss of plant biomass due to herbivory. The mechanism of action might be because of improved antioxidants production in AMF and *Rhizobium* inoculated plants upon herbivory for protecting chlorophyll from herbivore-induced oxygen damage.

CONCLUSION

The results revealed that chlorophyll and biomass contents of blackgram plants inoculated either singly or jointly with AMF and *Rhizobium* were greater over uninoculated plants. Upon exposure to *S. litura*, these plant parameters reduced significantly in all the treatments except the treatments inoculated with AMF. These results indicate that AMF and *Rhizobium* inoculation could minimize chlorophyll damage and plant biomass production during herbivory. Thus, AMF alone or in combination with *Rhizobium* could act as a bioprotective agent and be a part of the integrated pest management system.

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