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RESEARCH ARTICLE

Changes in Leaf Phenolics and Chlorophyll Content of Maize Inoculated with *Glomus intraradices* Upon *Spodoptera frugiperda* Infestation

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ABSTRACT

Maize, the third important cereal crop, is severely affected by *Spodoptera frugiperda* (fall armyworm). Arbuscular mycorrhizal fungi (AMF) colonized crops are shown to tolerate insect infestation because of systemic priming of defence against insect attack. The present study aimed to examine the effect of various doses of AMF (*Glomus intraradices*) inoculation on triggering tolerance against *S. frugiperda* infestation in maize. The total phenolics and the chlorophyll content of insect uninfested leaves obtained from AMF applied and insect attacked treatments were higher over AMF uninoculated plants upon insect attack. However, a greater concentration of phenolics and chlorophyll was observed in plants treated with maximum spore inoculum of 5 per seed. Similarly, plant biomass was also greater in the same treatment even after *S. frugiperda* infestation. These observations indicate the importance of AMF in sustaining maize growth and health during *S. frugiperda* attack.

Keywords: *Mycorrhiza; Plant defence; Fall armyworm; Phenolics and Chlorophyll*

INTRODUCTION

Severe outbreaks of pests and -causing organisms are currently observed due to climate change (Skendzic *et al*., 2021). According to United Nations Food and Agricultural Organization's report (2019), about 40% of food crops are lost every year due to insect infestation (Kaul *et al*., 2019). In maize alone, a yield loss of 21 to 53% per annum was reported due to *Spodoptera frugiperda* infestation alone (De Groote *et al*. 2020). Hence, effective measures are essential to control this polyphagous insect. The current global situation further warrants eco-friendly control measures over chemical insecticides.

Plants harbor numerous beneficial and harmful (Barber *et al*., 2013) microorganisms. Recent reports of plant microbiomes and the effect of endophytes on plant growth and health under biotic and abiotic stresses emphasized the importance of microbial endophytes (Porter *et al*., 2020; Singh *et al*., 2022). The endophytes were found to improve plant growth through nutritional and non-nutritional modes (Morelli *et al*., 2020). Further, endophytes-mediated increase in stress tolerance due to induced systemic resistance (ISR) and systemic acquired resistance (SAR) was observed in many crops (Khare *et al*., 2018). Arbuscular mycorrhizae are important endophytes that improve plant growth by enhancing the nutrient uptake

potential of plants as well as by modifying plant morphology (callose, trichomes, waxes, cuticle), and physiology (defence enzymes, antifeeding compounds, antioxidants) during herbivore attack (Lokhandwala and Hoeksema, 2019; Goddard *et al*., 2021).

The phenolics are important plant defence metabolites, which act as antioxidants and feeding deterrents of herbivores. Quinones, the derivatives of phenolics bind with proteins and minimize their accessibility to insects (Singh *et al*., 2022). Chlorophyll is an important photosynthetic pigment directly linkedto plant biomass production (Hu *et al*., 2015). During herbivore attack, plants produce reactive oxygen species (ROS), namely superoxide radical, singlet oxygen, hydroxyl radical, and hydrogen peroxide. Over-production of ROS upon herbivore attack damages chloroplast, affecting plant growth negatively. Inoculation of AMF increases plant photosynthetic efficiency (Gupta *et al*., 2021) and chlorophyll content (Mathur *et al*., 2018) during insect attacks.

However, there is a paucity of information about AMF mediated tolerance against *S. frugiperda* attack in maize. In this context, this study was performed to find out the effect of different doses of AMF (*Glomus intraradices*) inoculum on root colonization AMF induced changes in phenolics, chlorophyll, and biomass content of maize upon *S. frugiperda* attack.

MATERIAL AND METHODS

Plant, herbivore, AMF used for the study

Maize seeds (COH6) were obtained from the Department of Millets, Tamil Nadu Agricultural University (TNAU), Coimbatore. *S. frugiperda* larval egg mass (Accession number: NOC-03) was purchased from National Bureau of Agricultural Insect Resources (NBAIM), Bangalore, India and grown in the plastic container up to the second instar using maize leaves as a feed. AMF culture (*Glomus intraradices*) was obtained from the Department of Agricultural Microbiology, TNAU.

Experimental design

A pot experiment was conducted in the Department of Agricultural Microbiology, TNAU, Coimbatore, India with six treatments and three replications. AMF spores were recovered from commercial inoculum by the wet and decanting method (Gerdemann and Nicolson 1963). Individual AMF spore was isolated using capillary tube. The surface sterilized (with 1.6% sodium hypochlorite) maize seeds were coated using carboxy methyl cellulose with different doses of AMF spore as prescribed below. T_1- without spore (0), T_2- with one spore (1S), T_3- with two spores (2S), T_4- with three spores (3S), T_5-with four spores (4S), T_6-with five spores (5S). The spore-treated maize seeds were sown in mud pots (22×20×20 cm) containing sterile soil and sand (3:1). The pots were irrigated once in two days (100 mL pot^{-1}) with sterile water and once in five days with Hoagland's nutrient solution (50 mL pot^{-1}). After 9, 19 and 29 days of germination (DAG) maize plants were fed with *S. frugiperda* (2^{nd} instar and 2 larvae $plant^{-1}$) for 24 h. Uninfested leaf samples were collected from insect-attacked plants and changes in metabolites content were determined by analyzing total phenolics, chlorophyll, and biomass content. The uprooted plants were used for evaluating the mycorrhizal colonization percentage in roots.

Examination of arbuscular mycorrhizal infection in maize

Maize plants were uprooted after 10, 20, and 30 days of germination; to remove soil particles adhering to the root system, the root portion was washed gently in tap water, blot dried, and cut into 2 cm root bits. Then, the root bits were soaked in 10% potassium hydroxide (KOH) and incubated in a boiling water bath for 10 min. After incubation, KOH was poured off and 2% hydrochloric acid (HCl) was added, and then washed thoroughly with tap water. Then, the roots were stained with 0.008% trypan blue and incubated overnight Giovannetti and Mosse, (1980). Then, the root bits were observed under the low power objective of a light microscope (Magnus MLX-B Plus) and the percent mycorrhizal infection was calculated.

$$\text{Mycorrhizal colonization \%} = \frac{\text{Number of infected roots}}{\text{Number of roots observed}} \times 100$$

Estimation of total phenolics content of leaves

The fresh leaf sample (0.5 g) was ground in pestle and mortar with 80% methanol (1 mL) and centrifuged at 10,000 g for 10 min under refrigerated condition. Of the total quantity of supernatant collected, 0.2 mL was mixed with 1 mL of 1 N Folin-Ciocalteu reagent and 1 mL distilled water and incubated at room temperature for 30 min. After incubation, 1 mL of 20% sodium carbonate was added and incubated in water bath (100˚C) for one minute. After that, the absorbance was measured at 725 nm in spectrophotometer (Spectramax® i3X, California, USA) and the activity was expressed as mg gallic aicd g^{-1} fresh weight (FW) () (Baba and Malik, 2015).

Estimation of total chlorophyll content of leaves

100 mg fresh leaf sample was ground in 80% acetone (5 mL) and centrifuged at 3,000 g for 10 min at refrigerated condition. This procedure was repeated until the biomass becomes colourless. The final volume of the supernatant was made up to 10 mL using acetone (80%). The absorbance of the supernatant was determined at 663 and 645 nm in spectrophotometer (Li *et al*., 2019) and the total chlorophyll content was calculated as per the equation given below.

$$\text{mg chlorophyll a/g tissue} = 12.7(\text{A663}) - 2.69\,(\text{A645}) \times \frac{\text{V}}{1000 \times \text{W}}$$

$$\text{mg chlorophyll b/g tissue} = 22.9(\text{A645}) - 4.68\,(\text{A663}) \times \frac{\text{V}}{1000 \times \text{W}}$$

$$\text{mg total chlorophyll/g tissue} = 20.2(\text{A645}) - 8.02\,(\text{A663}) \times \frac{\text{V}}{1000 \times \text{W}}$$

A- Absorbance, V- final volume of cholorphyll extract, W- fresh weight of leaves sample

Estimation of plant biomass content of *S. frugiperda* attacked plants

After 24 h of *S. frugiperda* treatment, maize plants were uprooted and oven dried at 70°C for 3 days. After that, the dry biomass was calculated and expressed as gram plant^{-1}.

Statistical analysis

Statistical analysis was carried out with the software, Excel (version 2007) and SPSS (version 16.0). The values are represented as mean ± standard error of experimental data with a minimum of three replications. Duncan's multiple range test (DMRT) was performed at $P \leq 0.05$ for all the experiments.

RESULTS AND DISCUSSION

G. intraradices colonization in maize roots

Maize seeds were inoculated with different doses (1, 2, 3, 4 and 5 spores seed^{-1}) of *G. intraradices* spores and root infection percentage was assessed after 10, 20 and 30 days after seed germination (DAG) (Figure 1). Mycorrhizal infection was observed in all AMF inoculated plants and was absent in AMF spore uninoculated treatment. Percent mycorrhizal infection increased with an increase in spore load and the age of the crop. The highest mycorrhizal infection was noticed after 30 days of seed germination. During the initial days of crop growth (10 DAG), maximum mycorrhizal infection (35.38% ± 1.63, df = 12, P = 0.001, Table **1**) was noticed in treatment (T_6) where maize seeds were inoculated with 5 spores per seed (T_6). It was followed by T_5 (29.59% ± 0.35) and T_4 (23.44% ± 1.76). After 20 and 30 days of seed germination also, the same trend was observed. The highest percentage of mycorrhizal infection was observed in treatment T_6 where seeds were inoculated with 5 spores per seed (82.40% ± 1.67, df = 12, P = 0.001) after 30 DAG. Similarly, mycorrhizal infection and biomass production increased with an increase in AMF spore density in sugarcane (by Sivakumar, 2013). Zangaro *et al*., (2012) reported that there was a reduction in AMF colonization due to a decrease in AMF spore population in three Brazilian ecosystems. Mycorrhizal colonization percentage of plant roots was found to be directly linked with spore population (Suharno *et al*., 2017) as evidenced in the current study.

Total phenolics

To resist herbivore attacks, plants modify their physiology and synthesize various phenolic compounds. Systemic changes in primary and secondary metabolites content of plants infested with herbivore have been observed earlier (Pratyusha *et al*., 2022). Among various metabolites, phenolics act as inhibitors, toxicants, and parasitoids attractants against many insects (Singh *et al*., 2021). Dixit *et al*., (2017) observed significant increase in phenolics content in three different genotypes of cotton upon infestation by *Helicoverpa armigera* and *Spodoptera frugiperda*.

In this study, the phenolic content of *S. frugiperda* attacked plants increased with the age of the crop and the degree of mycorrhizal infection. After 10 DAG, there was no significant change in phenolic content between the control (T_1) and the mycorrhizal treatment like T_2 and T_3. However, the treatments like T_4, T_5 and T_6 recorded a greater quantity of phenolics, which was found to be statistically significant. Similarly, 20 and 30 DAG, the concentration of leaf phenolics increased with an increase in AMF spore density. The highest phenolic content (82.81 ± 3.41 mg gallic acid g^{-1} FW, df =12, P = 0.03, Table 2) was recorded in

leaf samples derived from *S. frugiperda* and mycorrhiza treated (T_6, 5 spores/seed) plants after 30 DAG. Similarly, *G. intraradice*s and *Rhizobium* inoculated blackgram plants were found to accumulate more phenolics than uninoculated control during herbivore (*Spodoptera litura*) attack (Selvaraj *et al*., 2020).

Chlorophyll content

Plenty of reports indicated photosynthesis and chlorophyll content of plants as primary physiological indicators of plant and herbivore interaction (Golan *et al.*, 2015, Mohammed *et al*., 2019). Mycorrhizae elicited accumulation of carotenoids, chlorophyll, anthocyanins, phenolics and nutrients against various stressors in different crops has been reported previously (Begum *et al*., 2019). In the current study, the chlorophyll content of the uninoculated control plant was on par with the chlorophyll content of leaves obtained from T_2, T_3 and T_4. However, statistically significant difference in chlorophyll content of plants of control with T_4 and T_5 was observed. This trend was similar in all the time intervals (10, 20 and 30 DAG). The highest concentration of chlorophyll a was observed after 30 DAG of plants with AMF application (T_6, 5 spores/seed) (1.11 ± 1.21 mg g^{-1} FW, df = 12, P = 0.13, Figure 2). Similarly, chlorophyll b content was also higher in T_6 (5S+SF) treatment (1.29 ± 0.32 mg g^{-1} FW, df =12, P = 0.06) compared to other treatments. The total chlorophyll content was higher in 5S+SF (2.40 ± 0.98 mg g^{-1} FW, df = 12, P = 0.05) treatment after 30 DAG compared to un-inoculated control (C+SF). Similarly, AMF (*Rhizophagus irregularis*) inoculated tomato plants reported (Formenti and Rasmann, 2019) to produce more chlorophyll over un-inoculated control during *Spodoptera littoralis* infestation. These observations revealed the importance of mycorrhizal infection in either preventing plants from biotic stress or mitigating the effect of biotic stress.

Biomass content

Mycorrhiza provides essential nutrients to colonized plants; thereby increasing the biomass content of the plants under stressed and unstressed conditions (Abdel-Salam *et al*., 2018). Similarly, in the present study inoculation of maize with AMF spores significantly increased the biomass content of maize. Maximum biomass recorded was 1.97 ± 0.01 g $plant^{-1}$ DW (df = 12, P = 0.03, Figure 3) in 5S+SF and the lowest biomass was observed in control with *S. frugiperda* treatment (1.25 ± 0.03 g $plant^{-1}$ DW) after 30 DAG.

Conclusion

Herbivore infestation results in differential modulation of the physiology of insect-infested and uninfected parts of a plant. Systemic induction of plant defense was noticed in many plants during insect attacks. Priming of this defense was evidenced in arbuscular mycorrhizal fungi colonized plants. During herbivory, plants are triggered to generate reactive oxygen species (ROS) which are responsible for denaturing macromolecules, enzymes, and organelles like peroxisomes and chloroplast. Arbuscular mycorrhizal fungal colonized plants are shown to tolerate the stress through greater production of antioxidants. In this study, we have analyzed the phenolics and chlorophyll content of insect uninfested parts of maize colonized with and without AMF (*G. intraradice*s). To our surprise, leaves from *S. frugipreda* attacked plants recorded greater concentrations of chlorophyll and phenolics. Despite *S. frugiperda* attack, AMF colonized maize and recorded more biomass production. These observations revealed that arbuscular mycorrhizal colonization (*G. intraradice*s) improves maize growth and imparts tolerance against *S. frugiperda* infestation by priming the defense systemically.

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Ethics statement

No specific permits were required for the described field studies because no human or animal subjects were involved in this research.

Consent for publication

All the authors agreed to publish the content.

Competing interests

There were no conflicts of interest in the publication of this content

Author contributions

Idea conceptualization-SR, TK, Experiments- SR, Guidance –TK, US, MM, RM, Resource- TK, US, Writing original draft – TK, SR, Writing- reviewing & editing- SR, TK

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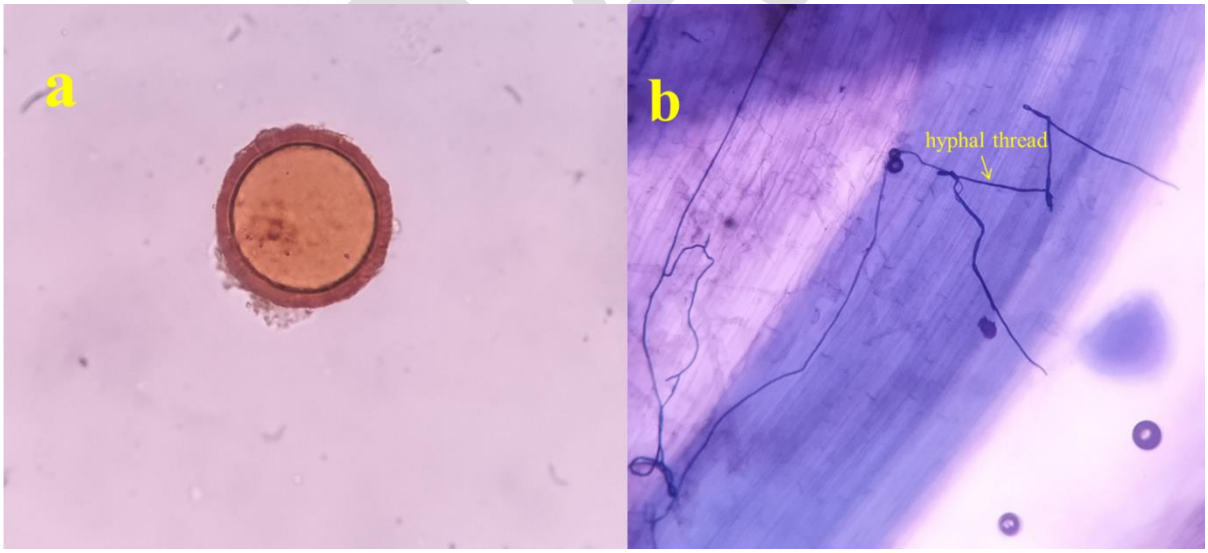

Figure 1. a) AMF (*G. intraradices*) spore used for inoculation, b) AMF root infection in maize plant (30 DAG)

Table 1. Degree of mycorrhizal colonization in maize roots inoculated with various doses of *G. intraradices* spores

Treatments	*G. intraradices* root infection (%)		
	10 DAG	20 DAG	30 DAG

T_1 (C+SF)	Nil	Nil	Nil
T_2 (1S+SF)	5.20±0.98^{e}	25.80±0.74^{e}	42.87±1.08^{e}
T_3 (2S+SF)	14.58±1.61^{d}	33.14±1.72^{d}	59.85±1.64^{d}
T_4 (3S+SF)	23.44±1.76^{c}	56.44±2.34^{c}	66.29±1.67^{c}
T_5 (4S+SF)	29.59±0.35^{b}	62.11±1.72^{b}	80.76±0.93^{b}
T_6 (5S+SF)	35.38±1.63^{a}	66.96±1.77^{a}	89.80±1.55^{a}
P	0.001	0.002	0.001

Values with different alphabets are significantly different according to Duncan's multiple range test ($P \leq 0.05$). C- control, DAG- days after seed germination, S- spore.

Table 2. Changes in leaf phenolics content of *G. intraradices* colonized maize upon S. *frugiperda* infestation

Treatments	Total phenolics (mg gallic aicd g^{-1} FW)		
	10 DAG	20 DAG	30 DAG
T_1 (C+SF)	18.65±1.76^{b}	19.35±1.07^{e}	19.65±0.02^{e}
T_2 (1S+SF)	18.60±3.48^{b}	28.19±1.26^{d}	33.69±0.03^{d}
T_3 (2S+SF)	19.44±1.15^{b}	33.19±1.32^{c}	66.03±0.81^{c}
T_4 (3S+SF)	21.40±2.08^{a}	61.28±0.01^{b}	78.82±1.72^{b}
T_5 (4S+SF)	21.73±0.98^{a}	62.40±2.41^{b}	81.65±2.98^{a}
T_6 (5S+SF)	22.65±1.42^{a}	67.31±1.73^{a}	82.81±3.41^{a}
P	0.02	0.001	0.03

Values with different alphabets are significantly different according to Duncan's multiple range test ($P \leq 0.05$). C- control, DAG- days after seed germination, S- spore

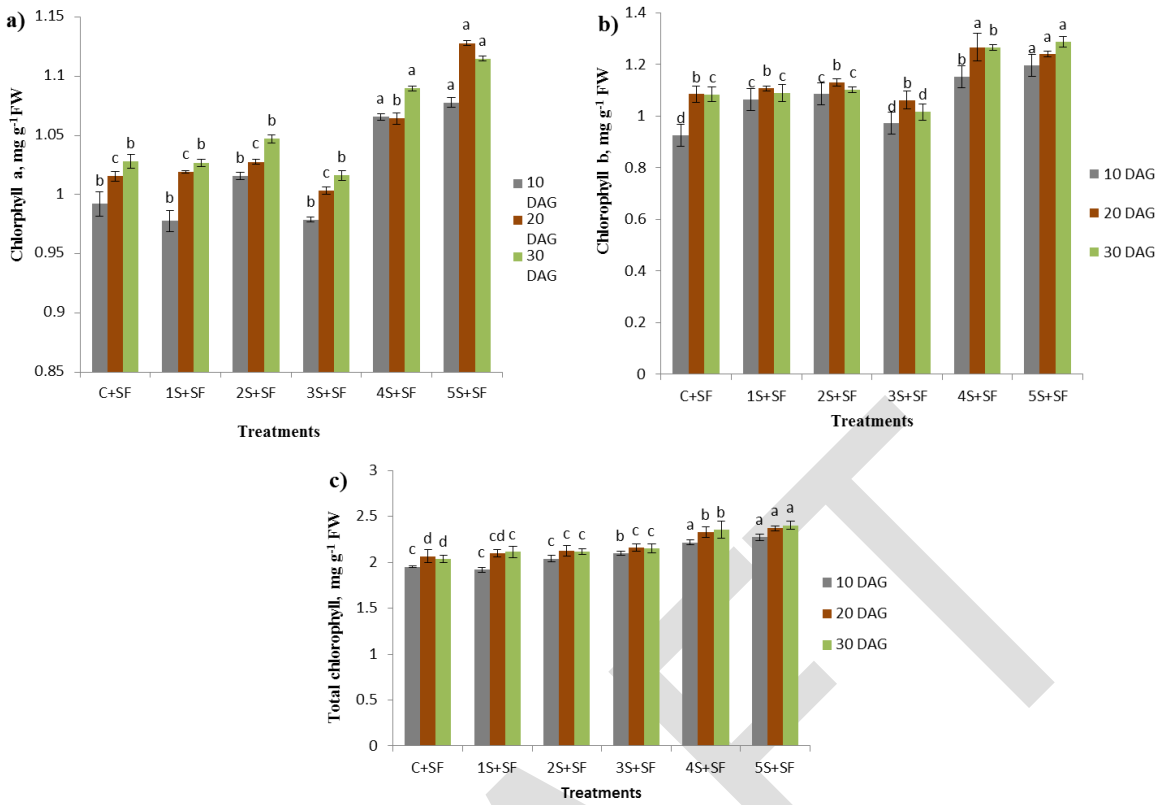

Figure 2. Changes in chlorophyll content of *G. intraradices* colonized maize upon S. *frugiperda* infestation. a) chlorophyll a, b) chlorophyll b, c) total chlorophyll. Values with different alphabets are significantly different according to Duncan's multiple range test ($P \leq 0.05$). C- control, DAG- days after seed germination, S- spore.

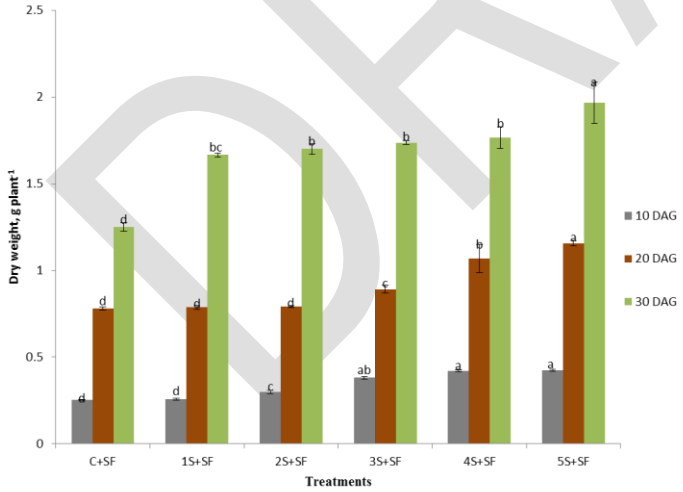

Figure 3. Biomass content of *G. intraradices* colonized maize upon S. *frugiperda* infestation. Values with different alphabets are significantly different according to Duncan's multiple range test ($P \leq 0.05$). C- control, DAG- days after seed germination, S- spore.