Effect of environmentally sustainable microbial fertilizer on Insect-induced plant responses

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Abstract

Application of Vesicular Arbuscular Mycorrhiza (VAM) is well known as an environmentally sustainable alternative to chemical fertilizers. However, its efficacy in fortifying plants against damage is still not known. We studied the effect of damage mechanically or by generalist caterpillar, Tobacco cutworm (*Spodoptera litura*), on soybean (*Glycine max*) was grown in soil containing VAM. Changes in chlorophyll, carotenoids, phenol and tannin were compared. We found that VAM not only facilitated improvement in growth parameters of soybean, but aided the increase in primary and secondary metabolites following damage. Thus, VAM may prove to be a promising environmentally-sustainable fertilizer in fields.

Key words: Chlorophyll a and b, Carotenoids, Phenols, *Spodoptera litura*, Tannins, Vesicular arbuscular mycorrhiza

Introduction

Microbial fertilizers are emerging as efficient growth promoters in sustainable agriculture systems. These beneficial soil microorganisms play an essential role in decomposing the organic matter of the soil and thereby release macro as well as micro nutrients necessary for plant growth (Van der Heijden *et al.*, 2008). Commonly used chemical fertilizers not only have a high processing cost but their excessive use can cause aggregation of soil nutrients. Moreover, there is a soil run-off pollution risk (Aktar *et al.*, 2009). Microbial fertilizers on the other hand are cost effective as well as help in releasing bound nutrients from the soil. Vesicular arbuscular mycorrhiza (VAM) is a fungal bio-fertilizer which acts as an obligate symbiont to many crops (Tylka *et al.*, 1991). VAM increases the soil nitrogen and phosphorus uptake in the host plant which influences their growth in exchange for its carbon requirements (Soka and Ritchie, 2014). It penetrates into the roots of the host and triggers a series of biochemical and

cytological events. These events are initiated by penetration of elicitor molecule in the host cell which further lead to series interdependent reactions such as activation of defense genes alteration of membrane permeability, activation of enzymes bound to plasma membrane and generation of small signaling molecules (Song *et al.*, 2011). These changes are vital for VAM establishment in host. However, penetration of a foreign microbe can induce production of metabolites in the plants that help the plant in defending against the microbe effects. Plant secondary metabolites are highly diverse in their structure and chemical nature. These metabolites, unlike traditional primary metabolites (carbohydrates, proteins, lipids, nucleic acids), do not directly play a role in the vital physiological functions of the plants. They are however, important for plant defence mechanisms (Jeaun *et al.*, 2005; Nicholson and Hammerschmidt, 1992; Maillard, 1996). Secondary metabolites are present in all higher plants and have a mutually exclusive distribution within the plant family (Wink, 2003). Phenolic compounds or polyphenols are a family of molecules widespread in the plant kingdom. This class of compounds includes phenolic acids (simple phenols), flavonoids, lignins, stilbenes, coumarins and tannins (Glombitza and Gerstberger, 1985;

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Harborne, 1980; Boros *et al.*, 2010). They are important for the defense mechanism in plants against pathogenic microbes as well as insect herbivores. These compounds elevate the activity of oxidative enzymes when the host cell is invaded (War *et al.*, 2012). Tannins also play an important role in plant defense against pathogens such as bacterial, fungal and nematode attack by accumulating on the area of invasion and thereby inhibiting their further development (Feucht *et al.*, 1997; Collingborn *et al.*, 2000). They are also reported to help against attack by insects and other herbivores, as the presence of tannins makes the plant less palatable for them (Mueller-Harvey and Mc Allan, 1992; Woodward and Coppock, 1995; Feucht *et al.*, 1997; Jean-Blain, 1998; Bennick, 2002).

Soybean (*Glycine max* [L.]) is a widely used leguminous crop belonging to Leguminosae (Fabaceae) contributing to 68% of the legumes worldwide (Naamala *et al.*, 2016). It is a rich protein and oil source and used as both foods as well as feed crop in livestock industry (Mateos-Aparicio *et al.*, 2008; Dei, 2011; Mapope and Dakora, 2016). However due to high protein content in its seeds, soybean requires a large amount of N and P (Fujikake *et al.*, 2003; Riaz, 2006). In order to obtain the optimum yield of soybean, it is important to use both nitrogen fixation in the nodules and inorganic nitrogen assimilation in the roots. Moreover, unavailability of free phosphorus causes further constraint in soybean cultivation. Thus, addition of VAM not only provides N fixation for the plants but also works as P- solubilizing bio-fertilizer, thereby improving N and P uptake in the plant. Studies have also shown VAM to help plant growth under drought and salinity stress (Aliasgharzad *et al.*, 2006; He *et al.*, 2007). However, whether VAM supplementation can also help the plant immunity against insect attack or whether biofertilizer-facilitated development results in immunity tradeoff, is still unclear. Therefore, we studied the effect of the microbial fertilizer VAM on induced responses against the generalist insect *Spodoptera litura* in soybean in terms of plant growth, primary and secondary metabolites. We further studied the variation in metabolite response against mechanical and insect injury in VAM supplemented soybean plants.

Material and methods

Plants: Soybean (*Glycine max*) variety Pusa-9712 seeds were obtained from Indian Agricultural Research Institute (IARI), New Delhi. Plants were grown in earthen pots of 6-inch diameter in an insect free plant enclosure at Sri Venkateswara college, New Delhi. Seeds were sown in supplementation with VAM in the ratio 10gm per kg of soil. Control plants were sown without any microbial bio-fertilizer supplementation. All the plants were measured for relative leaf count and shoot length after every two weeks.

VAM attachment to plants: VAM root attachments were observed in the plant during the first week of plant sowing. From the day the cotyledon growth was observed, three plants were removed from soil and the roots were washed with double distilled water to remove dust particles. These roots were then stained with lactophenol cotton blue stain for 5 min and observed under the microscope in order to verify VAM attachment.

Insects: *Spodoptera litura* were reared on castor leaves in 10 cm dia x 15 cm containers at Animal-Plant interactions lab, Sri Venkateswara College. The larvae were maintained at 28 ± 2 °C and 60-70 % RH. The adult moths were given 10 % honey solution fortified with multi vitamins.

Induction: The induction of the plants was performed when the plants reached stage 12 on the BBCH scale (Lancashire *et al.*, 1991). Overnight water satiated third instar *S. litura* were released on the 4th leaf of the plant in 4 cm dia x 2 cm plastic clip cages in 15 replicates on both VAM supplemented as well as un-supplemented control plants. The insects were left to feed on the plants for 24 h. Equal numbers of plants were injured on the 4th leaf with a punching machine on the fourth leaf for mechanically damaging the plants (Mithöfer *et al.*, 2005). The same number of undamaged plants from both VAM supplementation and plants grown in soil without VAM, received empty clip cages. The experiments were divided into short duration effects (24 h) and long duration effects (3 and 7 day). The test sets used were as follows: VAM supplemented plants without damage (VUN), VAM supplemented plants with mechanical damage (VM), VAM supplemented plants with insect damage (VI), control plants without damage (CUN), control

plants with mechanical damage (CM) and control plants with insect damage (CI).

Phenols and Tannin estimation: Phenol and tannin estimation was done at day 20 and day 34. Ten replicates were tested from each treatment. About 100 ± 0.03 mg samples of leaf were finely grounded in 10 times the volume of 80% ethanol. Samples were then centrifuged at $10,000 \times g$ at 4°C for 20 minutes. Supernatant was collected in a tube and evaporated at 50°C overnight. The residue was re-dissolved in 5 ml of water. The sample thus obtained was divided into two parts. Using 1N Folin – Ciocalteu reagent method, first part was used for phenol estimation and the second part was used for tannin estimation (Makkar *et al.*, 2007). Different concentrations from 10 -100 μg of Tannic acid was used as standard for the relative content estimation.

Chlorophyll and carotenoid estimation: Chlorophyll and carotenoid estimation was done at day 20 and day 34 in a set of ten replicates from each treatment set. About 0.1 gm of leaf was grounded till liquification in 10 mL of 80% acetone in dark (Arnon, 1949). The grounded sample was filtered with filter paper and absorbance was measured at 663, 645 and 470 nm. The calculations were done according to the following equations:

$$\text{Chl } a \left(\frac{\text{mg}}{\text{g}} \right) = \frac{[(12.7 \times A_{663}) - (2.6 \times A_{645})]}{\text{mg leaf tissue}} \times \text{ml of acetone}$$

$$\text{Chl } b \left(\frac{\text{mg}}{\text{g}} \right) = \frac{[(22.9 \times A_{645}) - (4.68 \times A_{663})]}{\text{mg leaf tissue}} \times \text{ml of acetone}$$

$$\text{Car}_{(\text{Carotene} \& \text{xanthophyll})} = \frac{1000A_{470} - 1.9 \times \text{Chl } a - 63.14 \times \text{Chl } b}{214}$$

Estimation of plant biomass: After removing the leaf samples, plants were collected individually in separate paper envelopes and were then heated at 100°C for 48h. The plants were again measured for their dry weight content and measured on weighing balance (Mettler Toledo, India).

Statistics: One-way ANOVA with Student-Newman-Keuls posthoc test was done using the SigmaPlot software (ver 12.0).

Results and Discussion

The root started growing approximately on the 5th day of sowing and VAM attachment was observed as early as 2nd day of root development which was observed about 7 days after sowing (figure 1). A significant height increase was observed in inoculated soybean seedlings after 20 and 34 days after sowing (figure 2). Height growth of inoculated soybean of 20 and 34 days was highly significant as compared to the non-inoculated plants. There was no significant difference in the number of leaves between inoculated and non-inoculated plants at day 20 of sowing. However, the number of leaves present in supplemented soybean at the end of 34 days was highly significant as compared to the non-supplemented plants ($F=183.134$, $P<0.001$) (figure 3). Dry matter of soybean growth of supplemented plant of 34 days was highly significant as compared to the non-supplemented plants. Thus, soybean plants with VAM interaction have significantly high dry matter than plants without VAM on the two days after sowing (figure 4). Both treatment ($F=81$, $P<0.001$) and day ($F=3.003$, $P<0.001$) had a significant effect on the phenol concentration. Phenol content significantly increased in 34 day old plants supplemented with VAM after 24h of damage, which remained elevated even after 3 days of damage, specifically in VI set. A slight increase in phenols with mechanical and insect damage (CM and CI) was also observed in control set of plants on day 3 of damage in the same age set (34 day old). No significant change in phenols of 20day old plants irrespective of treatment, was observed at 24h of damage ($P<0.001$). However, after 3 days of damage in 20 day old plants, a decrease in phenols was observed in VAM supplemented plants, especially in VUN and VI sets. In 20 day plants, tannin content decreased after 24h of insect damage ($P<0.001$), whereas in 34 day old plants, tannin content was significantly increased with both VAM supplementation as well as damage ($P<0.001$) (Table 2). Similar to phenols, tannin content also decreased in 20 day old plants compared with CUN after 3 days of damage irrespective of VAM supplementation ($P<0.001$). On the contrary in 34 day old plants tannins significantly increased after 3 days of damage irrespective of VAM supplementation ($P<0.001$).

Figure 1: (a) VAM attachment inside the root tissues of VAM supplemented plants stained with fungi specific stain lactophenol cotton blue and (b) Unstained roots of plants without VAM supplementation (control)

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Figure 2: Mean ($\pm$ S.E.) height of control and VAM supplemented soybean plants on day 20 and day 34 after sowing

Figure 3: Mean ($\pm$ S.E.) number of leaves in control and VAM supplemented soybean plants on day 20 and day 34 after sowing

Figure 4: Mean ($\pm$ S.E.) dry matter of control and VAM supplemented soybean plants on day 34 after sowing

Table 1: Concentration of phenols (day 1 and 3) (µg/g of leaf weight) using one way analysis of variance

	Day 1		Day 3	
	Means ± Standard error		Means ± Standard error	
	20 day	34 day	20 day	34 day
VUN	6.95 ^a ± 2.18	9.13 ^b ± 1.78	1.58 ^a ± 1.05	1.90 ^{ab} ± 1.17
VM	3.27 ^a ± 1.52	9.90 ^b ± 1.40	4.24 ^b ± 1.30	2.15 ^b ± 0.88
VI	4.87 ^a ± 2.11	8.01 ^b ± 1.27	1.43 ^a ± 0.90	4.19 ^c ± 0.14
CUN	5.62 ^a ± 1.94	2.82 ^a ± 1.85	8.20 ^c ± 0.87	0.49 ^a ± 0.49
CM	5.99 ^a ± 2.15	8.10 ^b ± 1.91	5.16 ^c ± 1.03	1.73 ^{ab} ± 1.06
CI	2.19 ^a ± 1.18	4.64 ^{ab} ± 2.00	0.74 ^a ± 0.69	1.18 ^{ab} ± 0.53
P	<0.001	<0.001	<0.001	<0.001
Significance	**	**	**	**

** : Significant and NS: Non-Significant.

Table 2: Concentration of tannins (day 1 and 3) (µg/g of leaf weight) using one way ANOVA

	Day 1		Day 3	
	Means± Standard error		Means± Standard error	
	20 day	34 day	20 day	34 day
VUN	3.68 ^b ± 1.03	4.37 ^c ± 0.70	9.54 ^a ± 6.33	1.89 ^b ± 1.20
VM	3.67 ^b ± 0.77	3.27 ^b ± 0.31	25.43 ^b ± 8.19	3.22 ^b ± 2.16
VI	2.71 ^{ab} ± 0.98	4.53 ^c ± 0.94	10.45 ^a ± 6.91	3.55 ^b ± 0.49
CUN	3.79 ^b ± 1.41	0.89 ^a ± 0.58	49.02 ^b ± 4.42	0.01 ^a ± 0.01
CM	4.64 ^b ± 1.13	4.67 ^c ± 1.25	37.86 ^b ± 7.36	0.84 ^b ± 0.52
CI	1.32 ^a ± 0.46	2.20 ^{ab} ± 0.87	5.56 ^a ± 5.56	2.33 ^b ± 1.26
P	<0.001	<0.001	<0.001	<0.001
Significance	**	**	**	**

** : Significant and NS: Non-Significant.

Table 3. Concentration of chlorophyll a (µg/g of leaf weight) at day 3 and 7

	Day 3		Day 7	
	Means± Standard error		Means± Standard error	
	20 day	34 day	20 day	34 day
VUN	1.34 ^b ± 0.34	0.24 ^a ± 0.07	0.83 ^c ± 0.23	0.41 ^a ± 0.19
VM	0.13 ^a ± 0.13	0.22 ^a ± 0.14	1.15 ^c ± 0.22	0.46 ^a ± 0.28
VI	0.36 ^{ab} ± 0.36	0.43 ^a ± 0.14	0.35 ^b ± 0.24	0.46 ^a ± 0.22
CUN	0.78 ^b ± 0.28	0.24 ^a ± 0.04	0.12 ^b ± 0.09	0.57 ^a ± 0.15
CM	1.10 ^b ± 0.30	0.29 ^a ± 0.13	0.38 ^b ± 0.18	0.75 ^a ± 0.10
CI	1.18 ^b ± 0.25	0.22 ^a ± 0.09	0.05 ^a ± 0.03	0.25 ^a ± 0.15
P	<0.001	0.043	<0.001	0.011
Significance	**	**	**	**

** : Significant and NS: Non-Significant

Table 4. Concentration of chlorophyll b ($\mu\text{g/g}$ of leaf weight) at day 3 and 7

	day 3		day 7	
	Means $\pm$ Standard error		Means $\pm$ Standard error	
	20 day	34 day	20 day	34 day
VUN	$0.67^c \pm 0.12$	$0.24^a \pm 0.06$	$0.45^c \pm 0.11$	$0.22^b \pm 0.08$
VM	$0.17^a \pm 0.05$	$0.43^a \pm 0.12$	$0.16^a \pm 0.08$	$0.232^{bc} \pm 0.13$
VI	$0.07^a \pm 0.13$	$0.22^a \pm 0.12$	$0.61^c \pm 0.10$	$0.045^a \pm 0.09$
CUN	$0.41^b \pm 0.12$	$0.21^a \pm 0.03$	$0.12^a \pm 0.07$	$0.295^{bc} \pm 0.05$
CM	$0.68^c \pm 0.14$	$0.22^a \pm 0.11$	$0.09^{ab} \pm 0.09$	$0.129^{ab} \pm 0.04$
CI	$0.55^b \pm 0.17$	$0.29^a \pm 0.08$	$0.23^b \pm 0.05$	$0.382^c \pm 0.07$
P	<0.001	<0.001	<0.001	<0.001
Signification	**	**	**	**

** : Significant and NS: Non-Significant.

Table 5. Concentration of carotenoid (xanthophyll & carotene) ($\mu\text{g/g}$ of leaf weight) at day 3 and 7

	Carotenoid (Xanthophyll & carotene) day 3		Carotenoid (Xanthophyll & carotene) day 7	
	Means $\pm$ Standard error		Means $\pm$ Standard error	
	20 day	34 day	20 day	34 day
VUN	$1545.86^b \pm 200.11$	$429.42^b \pm 118.56$	$1628.29^b \pm 873.29$	$619.35^{ab} \pm 280.60$
VM	$232.14^a \pm 175.48$	$297.56^a \pm 179.68$	$2383.27^b \pm 850.58$	$268.72^a \pm 195.67$
VI	$383.26^{ab} \pm 289.72$	$869.22^c \pm 232.48$	$644.30^{ab} \pm 880.491$	$513.85^{ab} \pm 184.47$
CUN	$894.80^b \pm 226.88$	$443.70^b \pm 93.16$	$430.62^{ab} \pm 747.43$	$703.83^b \pm 106.42$
CM	$1130.75^b \pm 229.77$	$404.35^b \pm 142.90$	$902.15^{ab} \pm 825.47$	$877.46^{bc} \pm 65.49$
CI	$1467.769^b \pm 220.39$	$427.26^b \pm 158.77$	$70.63^a \pm 95.57$	$287.49^a \pm 149.56$
P	<0.001	<0.001	<0.001	<0.001
Signification	**	**	**	**

** : Significant and NS: Non-Significant.

In 20 day old plants, VM showed a significant decrease, in chlorophyll *a* content after 3 days of damage whereas after 7 days of damage, chlorophyll *a* significantly decreased in insect damaged plants (both VI and CI) ($P < 0.001$) (Table 3). On the contrary, in 34 day old plants, no significant change in chlorophyll *a* was observed after 3 days and 7 days of damage. Chlorophyll *b* content, in 20 day VAM plants (VM and VI) was significantly decreased after 3 days of damage ($P < 0.001$). Chlorophyll *b* in this set of VM plants remained low even after 7 days of damage, whereas it significantly increased in 20 day old VI plants (Table 4). In contrast, in 20 day old CI plants

Chlorophyll *b* showed increase after 3 day of damage and subsided on day 7. 34 day old plants, showed no significant change in Chlorophyll *b* content after 3 days of damage, whereas it significantly decreased in VI on day 7 of damage. On the contrary 34 day old CI plants showed an increase in Chlorophyll *b* content after 7 day of damage ($P < 0.001$).

In 20 day old plants, significantly decreased carotenoids was observed in VM plants on 3rd day of damage, these levels however increased after 7 days of damage ($P < 0.001$) (Table 5). An overall increase was observed in VAM supplemented plants after day 7 of damage. In 34 day old plants, VM again showed decreased carotenoid ($P < 0.001$). However, VI plants showed significantly increased carotenoid content, which decreased by day 7. An overall decrease in carotenoid was observed in 34 day old plants by 7 day of damage.

Symbiotic association involves most plant species and certain fungal species which has great relevance to soil ecosystem functions, especially nutrient dynamics, microbial processes, plant ecology and agriculture. VAM occur naturally and are an important component of tropical soil system. Most of the plants form symbiotic relationship with mycorrhiza, which function as a bridge for the flow of energy and matter between plants and soils (Cardon and Whitbeck, 2011). Thus VAM performs beneficial functions for crops and other microorganisms such as phosphate-solubilizing bacteria (Panhwar *et al.*, 2009) and N_2 fixing bacteria (Naher *et al.*, 2009). In our study, VAM attachment to roots was observed as early at day 2 of root growth. VAM colonizes the host plant's roots inside the cortical tissues (Cardoso and Kuyper, 2006). The association may be either intracellular as in AMF, or extracellular as in ectomycorrhizal fungi. The root associated with the mycorrhiza have been reported to show increased active absorptive surface area and stimulate nutrient and water up take even in water stress condition (Dal Cortivo *et al.*, 2018).

Our results indicate that VAM supplementation positively affects the height of soybean after 20 and 34 days. A previous study also reported an increase in seedlings height upon inoculation with different strains of mycorrhizal fungi (Asghari *et al.*, 2005). During the first 20 days of sowing, there was no significant difference in number of leaves between

supplemented and non-supplemented plants. This could be due to the "lag phase" effect of VAM inoculation, where a lag phase is observed between mycorrhiza inoculation and the time period when its effect is manifested in the plant (Brindon and Shelton, 1993). The number of leaves growth of supplemented soybean at the end of 34 days was significantly increased as compared to the non-supplemented plants. These observations are supported by higher biomass production in VAM supplemented soybean possibly due to increased phosphorus uptake. Dry matter of soybean plant supplemented with VAM was significantly elevated compared to the non-supplemented plants, indicating positive influence of VAM inoculation. Similar observations of increased biomass due to VAM inoculation due to its ability of increased nitrogen phosphorus content to plants ultimately leads to increased production of pods, seeds and grain yield in crop plants (Asghari *et al.*, 2005; Caliskan *et al.*, 2008).

VAM supplementation also increased phenol content in soybean as early as 24h of insect damage indicating improved immune response to insect damage compared to mechanical damage in young plants (20 day old). On the other hand, 34 day old plant's immunity to mechanical damage also improved significantly upon VAM supplementation compared to control plants. However these elevated phenol levels decrease by day 3 in young plants (20 day old) whereas remain high in older plants (34 day old). This is possibly due to the anti-pathogenic properties of various phenolic compounds, leading to increased resistance (Nicholson and Hammerschmidt, 1992; Maillard, 1996). Phenols consist of a wide range of substances with an aromatic ring containing hydroxyl residues (OH-) and their functional derivatives which include the compounds such as catechol and protocatechuic acid, flavones, chlorogenic acid and quinones (King and Young, 1999). Together these compound form the constituent chemical barriers in plants against biotic stress (Nicholson and Hammerschmidt 1992). In contrast to phenols, tannin content did not increase significantly with damage in 20 day old VAM supplemented plants. However, it was observed to increase significantly in older plants with damage. According to Bennick (2002), soybean plant possesses sufficient amount of tannins in the natural state. The presence of tannins

makes them less palatable plants for herbivores and thus protects the plants from excessive predation. Tannins play an important role in plant defense as they accumulate at the wound sites on the plant in case of bacteria, fungi or nematodes, and thereby inhibit their development (Woodward and Coppock, 1995; Feucht *et al.*, 1997; Collingborn *et al.*, 2000).

Our results showed an increased chlorophyll *a* content in VAM supplemented plants (in both 20 and 34 day old plants) without damage (VUN) which remained higher even after day 7 of experimentation. However younger VAM supplemented soybean plants showed an increased chlorophyll *a* content after 7 days of mechanical injury faster in comparison to plants with insect damage.

In contrast, chlorophyll *b* content was not significantly affected by VAM supplementation and remained high in control plants. However, the control plants with damaged (CM and CI) showed increased chlorophyll *b* content in both 20 and 34 day old plants at both the time points. A previous report suggests the increasing of chlorophyll due to a greater carbon sink belowground and the improved nutrition of the host plants facilitated by symbionts, which further lead to higher rates of photosynthesis (Aseri *et al.*, 2008). Mycorrhiza have been reported to affect host plant by enhancing enzymatic production (Adriano-Anaya *et al.*, 2006), increase rate of photosynthesis (Wu and Xia, 2006), enhance nitrogen fixation in host (Antunes *et al.*, 2006) along with improving soil fertility (Charles *et al.*, 2006). Qurbanly *et al.* (2006) found a positive correlation between the nitrogen and chlorophyll content of leaves, mainly due to the presence of nitrogen in the structure of chlorophyll molecules. VAM significantly increased carotenoid content in younger plant stage with no damage whereas at old stages VAM plants were able to recover from insect damage by 3rd day which shows an improved plant response against insect herbivory. VAM supplemented plants also show an increase in the leaf carotenoid content. This is possibly due to increased nitrogen supply facilitated by VAM inoculation which is also observed in parsley (Cleward *et al.*, 2005). Moreover, nitrogen is the main constituent of all the amino acids. Correspondingly, an enhancement of protein synthesis and chloroplasts formation leads to an

increase in chlorophyll and carotene (Marschner, 1995). Our study shows that soybean alone or soybean-VAM interaction can induce plant systemic resistance against predators and pathogens.

Conclusion

VAM may be considered as an environmentally sustainable alternative to chemical fertilizers and may be considered safe for use in plants, not only for improving their growth but also for their defence against insects. Further, studies may be performed in order to determine the effect of VAM on plants in an ecosystem with multiple stress at various spatial and temporal levels.

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