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Research Article

Mycorrhizal Influence on Metabolite Constituents and Analysis of Phytochemicals by GC-MS in *Stevia Rebaudiana* (Bertoni) Bertoni

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Abstract

Arbuscular Mycorrhizal Fungi (AMF) are recognized to enhance the secondary metabolites in plants through symbiosis that maximizes the qualitative and quantitative metabolites in medicinal plants. In the present study more than 29 different types of compounds were detected by untargeted Gas Chromatography-Mass Spectrometry. Main components analysis clearly distinguished untreated roots of *Stevia rebaudiana* (Bertoni) Bertoni from roots treated either singly or in combination of three different AMF species (*Funneliformis mosseae*, *Glomus macrocarpum*, and *Gigaspora margarita*). The *S. rebaudiana* plants showed the highest level of metabolites like terpenoids, lipids and mineral nutrition in the mycorrhizal ones as compared to non-mycorrhizal ones. The phytochemical changes in the AMF infected plants in comparison to their respective controls by GC-MS analysis reported with higher levels of Terpenoids. The identified and quantified of measured active compounds was correlated with the expected influenced root colonization for the enhanced quantity of phytochemicals. The findings depicted that the mediated AMF improved the nutrient content and secondary metabolites in *S. rebaudiana* plants and may be used as an approaching source of important drugs in the field of pharmacology. It is concluded that AMF showed increase in phytochemical content and high N and P rates for root colonization in *Stevia rebaudiana* plant.

Key words- GC-MS, Secondary metabolites, *S. rebaudiana*

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Introduction

Plants are always impacted by microorganisms of which mycorrhiza are symbiotically associated with their roots, form mutualistic relationship (Dreher et al. 2019). Plants contain different phytochemicals constituents useful in the treatment of pharmaceutical industry. The plants and Arbuscular Mycorrhizal Fungi (AMF) mutualistic association benefited from transfer of nutrients (Lekberg

et al. 2016; Smith and Read 2008). Apart from acquisition of mineral nutrients AMF also provides quality plant products and contributes significantly for improvement of soil fertility (Castellanos-Morales et al. 2010; Baum et al. 2015; Wilson et al. 2009).

AMF also influences different secondary metabolites mediate interaction between host and AMF to colonize and establish AMF within the root tissues (Zhi-lin et al.

2007). Inoculation with AMF can improve plant health by enhancing the whole plant defense system and acquire resistance against pathogen attack (Chen et al. 2018). AMF enhances these metabolites in agricultural products and increases the quantity of the products (Hosseinzadeh et al. 2019).

Stevia rebaudiana (Bertoni) Bertoni is a subtropical perennial shrub that belongs to the Asteraceae family. There is growing interest in cultivation of *S. rebaudiana* because its leaves can be used to extract ent-kaurene diterpene glycosides, also known as steviol glycosides (SGs), which are naturally occurring sweeteners that are 250–300 times sweeter than saccharose (Gupta et al. 2016; Anton et al. 2010). In addition to being sweeteners it possesses anti-inflammatory, anti-hypertensive, anti-tumour, anti-diarrheal, immunomodulatory, and diuretic properties (Gupta et al. 2013; Bernal et al. 2011).

Screening of plant extracts is a new approach to find bioactive compounds in various plant species. Gas chromatography and mass spectrometry (GC-MS) is a combined analytical technique used to determine and identify different compounds present in a plant sample prepared in different solvents (Uma et al. 2009). GC-MS plays an essential role in the phytochemical screening of medicinal plants containing biologically active constituents. Therefore, the present study hypothesized that AMF inoculation would stimulate *S. rebaudiana* plants growth and enhances uptake of mineral nutrients. In the present investigation, an untargeted approach was applied to monitor the phytochemicals present in *S. rebaudiana* after treatment with different AMF species.

Material and Method

1. Plant identification and soil samples collection

Healthy plants of *S. rebaudiana* (Asteraceae) were purchased from the Shailesh Nursery, Kolhapur (MS.) India. The plant is maintained in Botanical Garden, Balwant College, Vita. The plant was authenticated, and voucher specimen (SP005) was deposited at Department of Botany, Balwant College, Vita. The rhizospheric and non-rhizospheric soil of plant up to the depth of 15 cm were collected in Ziplock polythene bags and brought immediately to the lab and used for isolation of AMF spores.

2. Isolation of AMF spores and Inoculum development

A method described by Gerdemann and Nicolson, (1963) was resorted for AMF spore isolation. Isolated, healthy, and identified AMF spores (*Funneliformis mosseae*, *Glomus macrocarpum*, and *Gigaspora margarita*) were taken for their mass multiplication and production of infective inoculum. The soil funnel culture technique (Subba Rao 1988) was followed for development of starter inoculum. Then starter inoculum (Trapped spores, soil samples, and infected roots) was used for mass multiplication using *Pennisetum purpureum* as host. After completion of set the root samples were checked for infectivity. This mixture of sand, soil, and infected root samples were served as native inoculum for the pot culture experiment.

3. Determination of AMF root colonization

For Root clearing and staining technique, a Philips and Hayman (1970) method was performed by 0.05% Trypan blue in Lactophenol, and percentage of AMF root colonization was estimated by the technique described by Nicolson (1960).

4. AMF inoculation and experimental set-up

In order to explore influence of AMF a pot culture experiments were performed in greenhouse conditions at the Botanical Garden of Balwant College, Vita (MS.) India. The experiment was set up as T-1: Control, T-2: *Funneliformis mosseae*, T-3: *Glomus macrocarpum*, T-4: *Gigaspora margarita*, T-5: Mix AMF (*F. mosseae* + *Gi. margarita*). The 35 cm diameter Polyethylene pots were filled 2 cm less than the regular filling for the AMF treatments except for the control. As treatments mentioned above, AMF inoculum of 200 gm per each pot were spread on the pots, then a layer of 2 cm soil was added to obtain regular filling, then 5 cm shoot tips of *S. rebaudiana* were sowed in each pot. The experiments were performed in triplicate.

5. Extraction

By the completion of the experiment after 90 days plants are harvested. The harvested plants of *S. rebaudiana* were shade dried and powdered using a mechanical grinder. The grounded powder was used for phytochemical extraction. The powdered samples of 10 gm was taken for extraction using ethanol (100ml) as a solvent in Soxhlet assembly to obtain 10% w/v concentration until complete extraction and the extract was used for GC-MS analysis.

6. GC-MS analysis

GC-MS analysis of ethanolic extracts of *S. rebaudiana* was carried out at SAIF, Shivaji University, Kolhapur using GC-MS (TQ8050). The method adopted for GC-MS analysis was conducted as the instrument built with a capillary column of 30 m length, 0.25 mm diameter, 0.25 μ m film thickness, column oven temperature 50 $^{\circ}$ C, and injection temperature 250.0 $^{\circ}$ C, column flow rate 1.01 mL/min. Identification of unknown compounds spectrum with those of spectral library and relative retention time (RRT) of the unknown peaks to the RRT of the reference standards.

Result and Discussion

Root Colonization

The *S. rebaudiana* inoculated with Mixed AMF (*F. mosseae* + *Gi. margarita*) showed substantial improvement in their root colonization over non inoculative ones (Table No. 1). Results showed that the higher hyphal root colonization rate was observed in *S. rebaudiana* roots inoculated with Mixed AMF treatment (*F. mosseae* + *Gi. margarita*) 65.33 $\pm$ 5.90 %, followed by *Gi. margarita* (60.33 $\pm$ 5.36%), *G. macrocarpum* (57.67 $\pm$ 6.77%), and *F. mosseae* (46.00 $\pm$ 1.15%). A significant decline in the AMF root colonization observed in the control.

In the present study AMF strains from natural habitat showed that local strains, especially *Gi. margarita*

compared to *F. mosseae*, and *G. macrocarpum* were more infective and rapidly colonize the root system of *S. rebaudiana*. The results also indicated that these AMF partners would not see be specific, even if there was variability between the colonization rate recorded in *S. rebaudiana*. Therefore, *S. rebaudiana* could establish symbiosis with several AMF species that differ in ecology, and the plants should be less specific to AMF in control condition. The present investigation demonstrated the mycorrhizal effectiveness of local AMF species and suggested promising perspectives for the application in the form of biofertilizer for sustainable agriculture.

Nitrogen and Phosphorus content

Inoculation of AMF significantly augmented by N $3.13 \pm 0.101\%$ and P $0.15 \pm 0.120\%$ content as compared to control $2.55 \pm 3.214\%$ and $0.10 \pm 1.010\%$ respectively. The major improvement of N and P uptake was obvious because of AMF inoculations. Mycorrhizal plants have been reported to have high level of N, P and K compared to non-AMF plants (Shi et al. 2021). According to Zou et al. (2021) AMF could stimulate the production of secondary metabolites like phenol, flavonoid, and carotenoid in plants where these compounds had antioxidant properties. AMF hyphae could penetrate deep into the soil and acquire nutrients from a large soil volume that would improves the nutrient status of plants. The effects of AMF inoculation increase in different parameters of plant growth were highly significant.

Table no. 1 Effect of AMF inoculum treatments on % root colonization, Nitrogen and Phosphorus content of *S. rebaudiana*:

Treatments		% root colonization			Nitrogen Content (%)	Phosphorus Content (%)
		Hyphal	Arbuscular	Vesicular		
T-1	Control	0.00 ± 0.00^a	0.00 ± 0.00^a	0.00 ± 0.00^a	2.55 ± 3.214^a	0.10 ± 1.010^a
T-2	<i>F. mosseae</i>	46.00 ± 1.15^{ab}	23.67 ± 6.94^a	21.00 ± 6.43^b	2.59 ± 4.235^a	0.12 ± 1.236^a
T-3	<i>G. macrocarpum</i>	57.67 ± 6.77^b	24.33 ± 3.76^{ab}	31.00 ± 5.29^b	2.63 ± 1.132^a	0.11 ± 0.010^a
T-4	<i>Gi. margarita</i>	60.33 ± 5.36^b	26.33 ± 5.04^{ab}	0.00 ± 0.00^a	2.98 ± 2.341^a	0.14 ± 3.215^a
T-5	Mix AMF (<i>F. mosseae</i> + <i>Gi. margarita</i>)	65.33 ± 5.90^c	38.67 ± 1.20^b	30.67 ± 7.22^b	3.13 ± 0.101^b	0.15 ± 0.120^b
AVG		59.83	28.25	27.55	2.79	0.124
P-value		0.001	0.046	0.010	0.049	0.001
S.E. (Mean)		3.1701	2.6125	3.6474	0.3846	0.0090
S.E. (diff)		5.3624	6.2574	7.3696	16.1467	10.8270
C.D. at 5%		11.9482	13.9424	16.4205	19.7458	23.5786
CV %		23.5206	39.4221	15.004	55.3647	21.5094

Qualitative and Quantitative phytochemical analysis by GC-MS analysis

A total of 29 compounds were identified from GC-MS analysis of ethanol fraction of *S. rebaudiana* exhibiting different phytochemical constituents. The chromatogram is presented in Fig, no 1 to 5, while chemical constituents with their Retain Time (RT) and concentration (%) were presented in Table no. 2. The bioactive compounds present in the GC-MS analysis carried out on ethanol fraction of *S. rebaudiana* were 1,1,6-trimethyl-3-methylene-2-(3,6,9,13-tetramethyl10,14-dimethylene-pentadec-4enyl)cyclohexane; 1HCycloprop [e]azulen-7-ol,decahydro-1,1,7-trimethyl-4-methylene1ar-(1a.alpha.,4a.alpha.,7.beta.,7a.beta.,7b.alpha.)-; 2,2,6-Trimethyl-1-(3-methylbuta-1,3-dienyl)-7-oxabicyclo[4.1.0] heptan-3-ol; 3,7,11,15-Tetramethyl-2-hexadecen-1-ol; 4,11,11-trimethyl-8methylene, [1R-(1R*,4Z,9S*)]-; 4,6,10,10-Tetramethyl-5-oxatricyclo[4.4.0.0(1,4)]dec-2-en-7-ol; 4,8,13-Cyclotetradecatriene-1,3-diol, 1,5,9-trimethyl-12-(1-methylethyl)-; 4-Hepten-3-one, 5-methyl-; 6-Undecyl-5,6-dihydro-2H-pyran-2-one; Acetic acid, 3,7,11,15-tetramethyl-hexadecyl ester; Bis(2-ethylhexyl) phthalate; Cyclohexane, 1,5-diisopropyl-2,3-dimethyl-; Dibutyl phthalate; Diisooctyl phthalate; Dotriacontane,

1-iodo-; Eicosane; Formic acid, 1-methylethyl ester; Heptadecane, 2,3-dimethyl-; Hexadecanoic acid, ethyl ester; Kaur-16-en-18-oic13-hydroxy-, methyl ester, (4.alpha.)-(./-.)-; Neophytadiene; Octacosane; Octadecane, 5-methyl-; Octadecanoic acid, ethyl ester; Phytol; Tetradecane, 4-methyl-; Thunbergol; trans-3-Methyl-4-octanolide; and Triacontane, 1-bromo.

Phytochemical screening of aqueous ethanol fraction of *S. rebaudiana* revealed presence of 29 different phytocompounds that have been documented to some other activities. Terpenoid compounds found to be more significant in all treatments (1,1,6-trimethyl-3-methylene-2-(3,6,9,13-tetramethyl10,14-dimethylene-pentadec-4enyl)cyclohexane; 1HCycloprop [e]azulen-7-ol,decahydro-1,1,7-trimethyl-4-methylene1ar-(1a.alpha.,4a.alpha.,7.beta.,7a.beta.,7b.alpha.)-; 2,2,6-Trimethyl-1-(3-methylbuta-1,3-dienyl)-7-oxabicyclo[4.1.0] heptan-3-ol; 3,7,11,15-Tetramethyl-2-hexadecen-1-ol; 4,11,11-trimethyl-8methylene, [1R-(1R*,4Z,9S*)]-; 4,6,10,10-Tetramethyl-5-oxatricyclo[4.4.0.0(1,4)]dec-2-en-7-ol; 4,8,13-Cyclotetradecatriene-1,3-diol, 1,5,9-trimethyl-12-(1-methylethyl)-; 4-Hepten-3-one, 5-methyl-; 6-Undecyl-5,6-dihydro-2H-pyran-2-one; Kaur-16-en-18-oic13-hydroxy-, methyl ester, (4.alpha.)-(./-.)-; Thunbergol). It was followed by lipids (Acetic acid, 3,7,11,15-

tetramethyl-hexadecyl ester; Dotriacontane, 1-iodo-; Eicosane; Octacosane; Octadecanoic acid, ethyl ester, and Tetradecane, 4-methyl-). The results agree with Olivia et al. (2017), who reported that highest concentration of terpenoids in *Hibiscus asper* indicates its numerous medicinal properties. Among the identified compounds Neophytadiene a lipid appeared in four treatments with varying % area and possesses Anti-inflammatory, Antimicrobial, Antioxidant, and Cardioprotective properties Gonzalez-Rivera (2023).

Similarly, A terpenoid 3,7,11,15-Tetramethyl-2-hexadecen-1-ol contain Antimicrobial, Antioxidant, and Anticholinesterase properties (Nazir et al. 2021). It was followed by Phthalate, Saturated hydrocarbon, Halogenated hydrocarbons, lactone, alkanes, volatile organic compounds and lactone fatty acid derivatives. Kumar et al. (2010); Olivia et al. (2021) reported that the major volatile compounds viz. phenols, esters, alkanes, aldehydes, ketones present in *Hibiscus asper* which had pharmaceutical properties.

Table no. 2: Effect of AMF inoculum treatments on qualitative and quantitative phytochemicals by GC-MS analysis of *S. rebaudiana*:

Sr. no.	Name of the compound	Area %				
		T-1	T-2	T-3	T-4	T-5
1	1,1,6-trimethyl-3-methylene-2-(3,6,9,13-tetramethyl-10,14-dimethylene-pentadec-4-enyl) cyclohexane	42.55	56.02	71.37	42.55	-
2	1H-Cycloprop[e]azulen-7-ol, decahydro-1,1,7-trimethyl-4-methylene-, [1ar-(1a.alpha.4a.alpha.,7.beta.,7a.beta.,7b.alpha.)]-	-	-	-	-	0.37
3	2,2,6-Trimethyl-1-(3-methylbuta-1,3-dienyl)-7-oxabicyclo [4.1.0] heptan-3-ol	-	-	-	-	13.13
4	3,7,11,15-Tetramethyl-2-hexadecen-1-ol	-	5.56	-	2.56	3.92
5	4,11,11-trimethyl-8-methylene-, [1R-(1R*,4Z,9S*)]-	-	-	-	-	0.13
6	4,6,10,10-Tetramethyl-5-oxatricyclo [4.4.0.0(1,4)] dec-2-en-7-ol	-	-	-	-	1.10
7	4,8,13-Cyclotetradecatriene-1,3-diol, 1,5,9-trimethyl-12-(1-methylethyl)-	-	-	-	8.61	-
8	4-Hepten-3-one, 5-methyl-	-	-	-	-	0.35
9	6-Undecyl-5,6-dihydro-2H-pyran-2-one	3.55	-	-	-	-
10	Acetic acid, 3,7,11,15-tetramethyl-hexadecyl ester	-	-	-	-	0.13
11	Bis(2-ethylhexyl) phthalate	-	-	-	-	0.52
12	Cyclohexane, 1,5-diisopropyl-2,3-dimethyl-	-	-	6.14	-	-
13	Dibutyl phthalate	-	20.11	-	13.82	-
14	Diisooctyl phthalate	-	-	-	1.75	-
15	Dotriacontane, 1-iodo-	-	-	-	2.00	-
16	Eicosane	-	-	-	-	0.13
17	Formic acid, 1-methylethyl ester	-	-	10.49	-	-
18	Heptadecane, 2,3-dimethyl-	-	-	-	0.64	-
19	Hexadecanoic acid, ethyl ester	-	-	-	-	0.78
20	Kaur-16-en-18-oic13-hydroxy-, methyl ester, (4. alpha.)-(./-.)-	31.05	-	-	-	-
21	Neophytadiene	-	18.31	12.00	18.96	13.61
22	Octacosane	-	-	-	1.08	-
23	Octadecane, 5-methyl-	-	-	-	-	0.17
24	Octadecanoic acid, ethyl ester	-	-	-	-	1.58
25	Phytol	-	-	-	2.15	2.85
26	Tetradecane, 4-methyl-	-	-	-	1.60	-
27	Thunbergol	-	-	-	3.65	-
28	trans-3-Methyl-4-octanolide	-	-	-	0.52	-
29	Triacotane, 1-bromo	-	-	-	0.60	-

The key role of AMF was to increase the qualitative and quantitative yield of phytochemicals particularly essential oils (Hazzoumi et al. 2015). In present study, not only quantitative phytochemicals affected by AMF, but also chemical profile of phytochemicals was altered. Terpenoids and lipids were the main phyto components among all known compounds in all the treatments.

Inoculation of *Gi. margarita* singly further enhanced the number of these compounds. The present study confirms the AMF symbiosis in the synthesis and % of individual compounds in *S. rebaudiana* (Table no. 2 and Fig. no 1 to 5)

Earlier studies reported that AMF increases the production of phytochemicals of medicinal and aromatic

plants (Begum et al. 2021; Weisany et al. 2017; Fokom et al. 2019). Furthermore, current studies were reported that AMF inoculations increase the Terpenoids and lipids content in *S. rebaudiana* medicinal plants. Results of Dreher et al. (2019) and Begum et al. (2021) showed that AMF inoculations resulted in the improvement of

phytochemicals and increased growth of plants. The present study exhibited that AMF inoculations enhanced the terpenoid content in *S. rebaudiana* plants. Terpenoids and lipids played an important role of mycorrhization, growth regulations in *S. rebaudiana* by competition, signalling, and nutrient uptake.

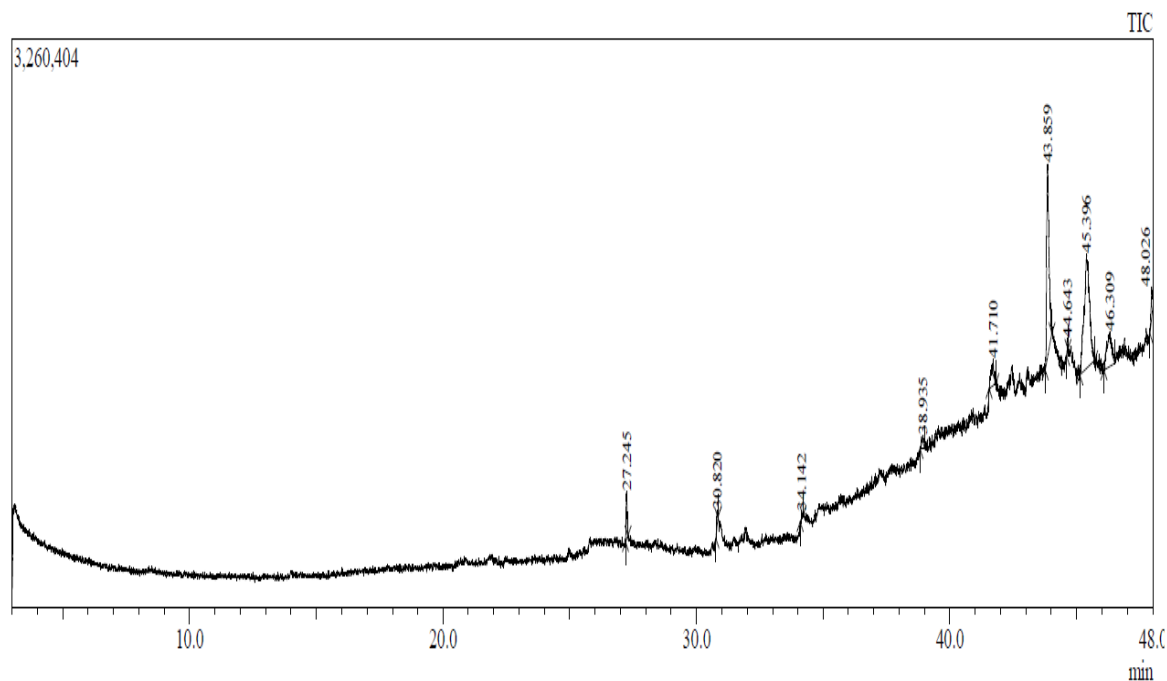


Fig. no 1: GC-MS chromatographic profile of ethanolic extract of non-inoculated *S. rebaudiana* (T-1)

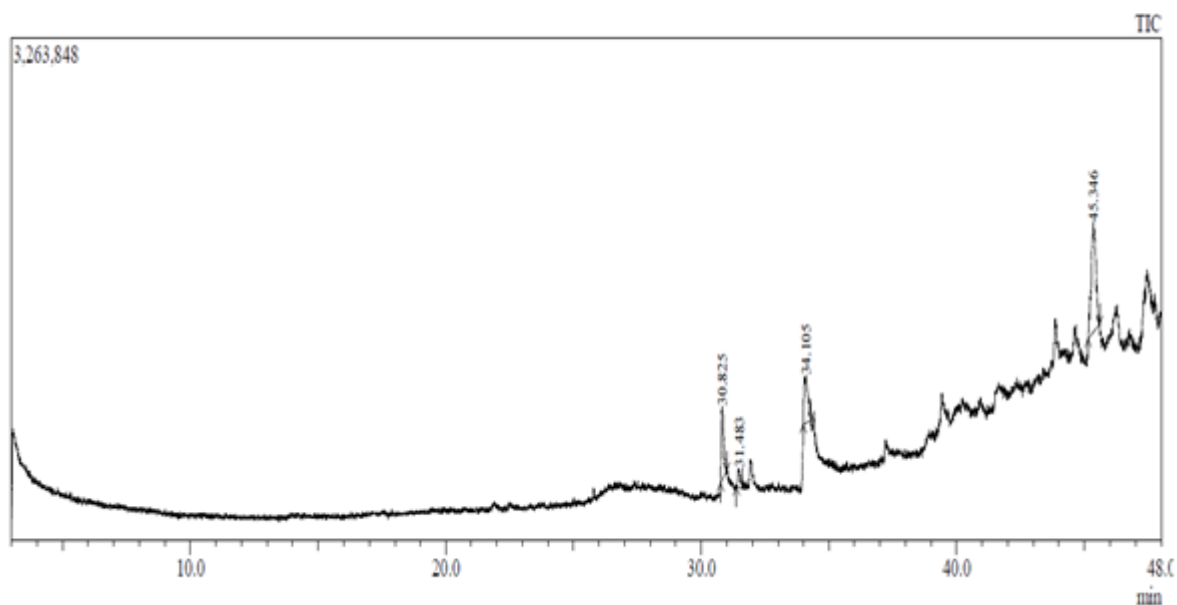


Fig no. 2: GC-MS chromatographic profile of ethanolic extract of *S. rebaudiana* inoculated with *F. mosseae* (T-2)

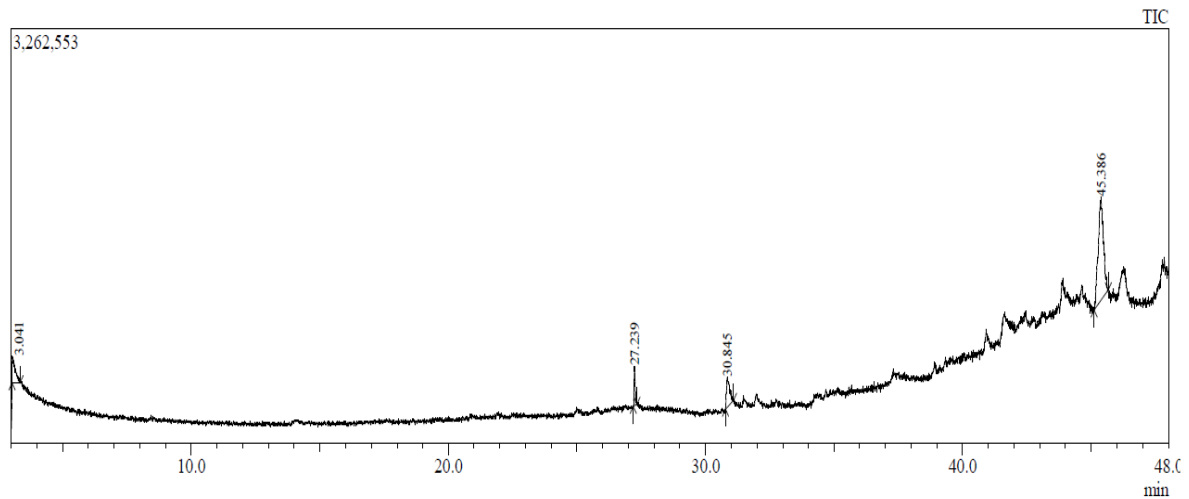


Fig no.3 GC-MS chromatographic profile of ethanolic extract of *S. rebaudiana* inoculated with *G. macrocarpum* (T-3)

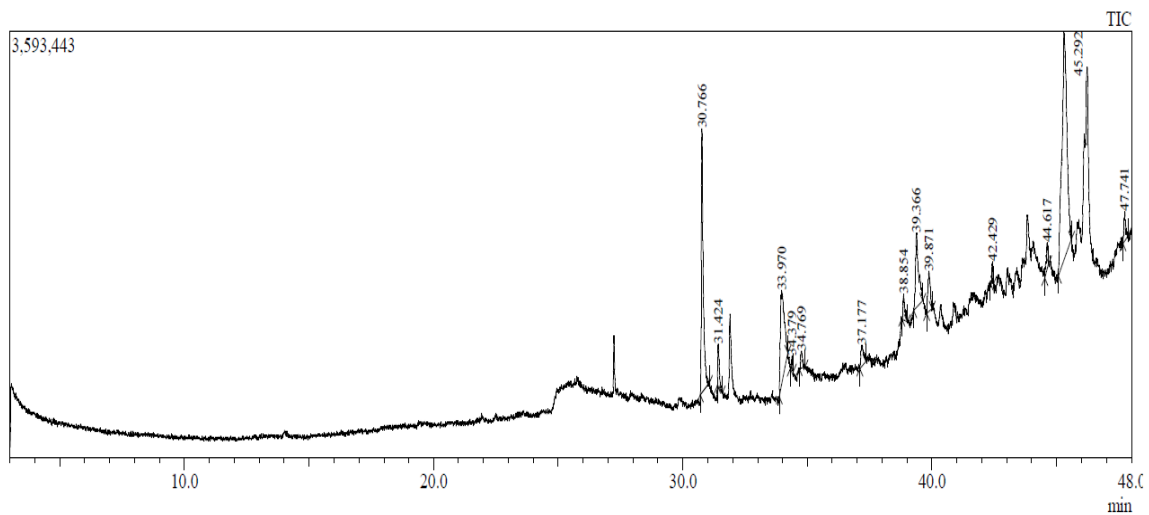


Fig no. 4: GC-MS chromatographic profile of ethanolic extract of *S. rebaudiana* inoculated *Gi. margarita* (T-4)

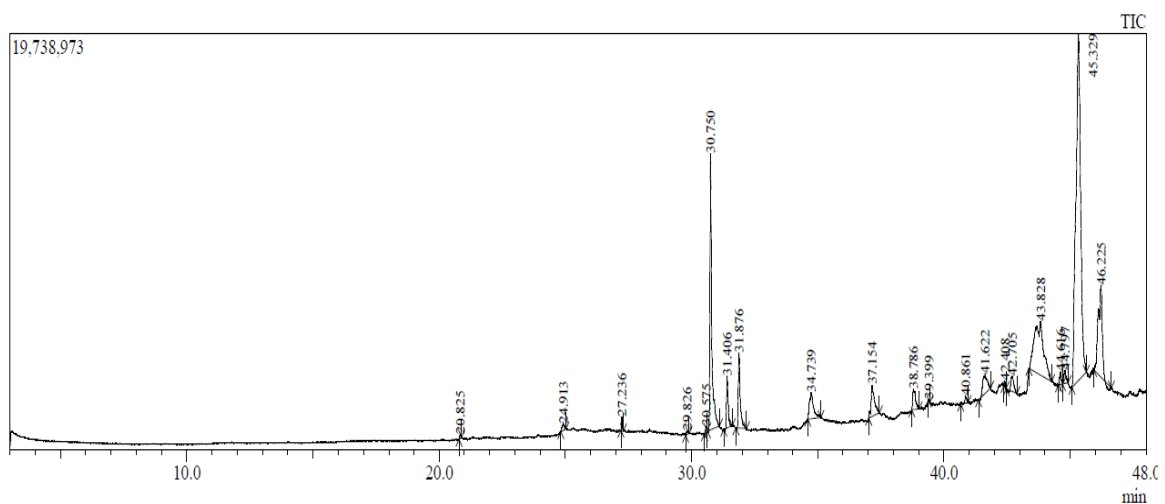


Fig no. 5: GC-MS chromatographic profile of ethanolic extract of *S. rebaudiana* inoculated Mix AMF (T-5)

These present findings were very consistent with the Begum et al. (2021) who studied that the enhanced phytochemicals which improve growth, essential oils, and secondary metabolites due to inoculation of AMF in *Nicotiana tabacum*, Rapparini et al. (2017) in *Artemisia*

annua, Ahmad et al. (2018) in *Cucumis sativus*. Therefore, in the present investigation, inoculation of AMF enhanced the presence of different phytochemicals may have impact on root colonization and nutrient

content (N and P) which leads to improved plant growth through stabilizing beneficial symbiosis.

Conclusion

The present findings clearly demonstrated that the role played by AMF in benefiting *S. rebaudiana* cultivation showed that AMF considerably improved the growth by improved root colonization, mineral nutrient uptake, and phytochemical. AMF improves both quality and productivity of phytochemicals in *S. rebaudiana* that was specifically noticed in the content of phytochemical compounds. The present findings suggested that the symbiotic association between AMF and *S. rebaudiana* plant may help to strengthen enhancement of phytochemicals. It is concluded that AMF had the potential to enhance phytochemicals constituents that may be used in industry as a prospective source of important drugs and for sustainable agricultural productivity

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