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Research Article

Pharmacognosy for efficient natural therapy

Changes in Growth Parameter and Essential Oil Composition of Ajwain (*Trachyspermum ammi*) Fruits in Response to Various Biofertilizers

Kamal Jeet[1,2] And Ashish Baldi[3]*

[1]*Research Scholar, I.K.G. Punjab Technical University, Jalandhar, Punjab, India-144603*

[2]*Career Point University, Hamirpur, H.P., India- 176041*

[3]*Department of Pharmaceutical Sciences & Technology, Maharaja Ranjit Singh Punjab Technical University, Bathinda, Punjab, India-151001*

Abstract: Unorganized natural nutrient cycle and depleted soil health may prove major limitation on the growth and development of medicinal plants and therefore quality and final yield of the cultivation. Therefore, studies concerned with the restoration of normal fertility to the soil and make it rich with certain nutrients are of the fundamental interest for medicinal plants. Herein, the present study was designed to investigate the multifaceted beneficial effects of (a) Phosphate solubilizing bacteria (*Pseudomonas fluorescens*) (b) Nitrogen-fixing bacteria (*Azotobacter chroococcum*) and (c) Mycorrhiza (*Sebacina vermifera*) on plant growth and seed essential oil composition of ajwain (*Trachyspermum ammi*). In this context, ajwain fruits were treated with selected biofertilizers to analyze the emergence pattern and to evaluate comprehensive growth and essential oil composition. About 25 days old seedlings were treated with selected biofertilizers. The results showed that all treatments had a good effect on seed emergence, though *Sebacina vermifera* assisted growth and essential oil production were much higher than other treatments. Concerning the composition, 13 compounds were identified using gas-chromatography. Major altered components were thymol, p-cymene and ϒ-terpinene. It is worthy to mention that each treatment proved to increase thymol only. The enhanced synthesis of thymol is supported by the speculation that biofertilizers may trigger an early or late step expression in biosynthetic pathway that leads higher production of thymol. As a conclusion, biofertilizers can alter the comprehensive growth including essential oil composition of ajwain, and also the chemical composition of ajwain was found to be strongly altered. Along with the study, *Sebacina vermifera* based biofertilizer indicated immense potentials to be utilize as multifaceted beneficial alternate for sustainable agronomical and medicinal plants productions.

Keywords: Ajwain, Azotobacter, Essential Oil, Plant growth, PSB, *Sebacina vermifera*

***Corresponding Author**

Dr. Ashish Baldi , Department of Pharmaceutical Sciences & Technology, Maharaja Ranjit Singh Punjab Technical University, Bathinda, Punjab, India-151001

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1. INTRODUCTION

By 2050, we will need to feed an additional 2 billion people due to the world's fast rising population.[1] The most important problem will be to supply the nutritional and medical demands of a growing population with the same amount of production area and water resources as now, while avoiding the use of pesticides and fertilizers of chemical origins. Pesticides and fertilizers of chemical origins have been proved to be deleterious to health human beings and for the environment. In such a difficult circumstance, we must seek out cost-effective and simple-to-implement solutions that will increase crop productivity in a sustainable and environmentally beneficial manner. Similarly, experts are currently proposing for the use of plant biologicals to boost plant production as a sustainable and environmentally beneficial approach. These biologicals benefit plants in a variety of ways, including enhancing soil quality, water retention, nutrient availability, and resistance to biotic and abiotic stress.[2-5] Mycorrhizal fungi are well-known plant biological agents, and the mycorrhizal association is the world's oldest symbiotic relationship between plants and soil fungi. This organism represents about 95% of all terrestrial plant species. Mycorrhizal fungi help host plants in a variety of ways, including nutrient facilitation, pathogen protection, drought stress tolerance, increased essential activity (photosynthesis), vegetation and reproduction.[6] Furthermore; mycorrhiza has been shown to reduce the effects of heavy metal toxicity. Mycorrhizal associations can range from obligatory to mutualistic, and the fungal symbiont may be limited to benefiting from the plant's carbon source or may engage in other activities such as mineralization of nutrients from non-living organic and inorganic sources.[6] Many mycorrhizal fungi are non-cultivable axenically outside the host plant in pure culture.[7] Whereas, *S. vermifera*, a member of the Sebacinaceae family, is cultivable outside the host plant and may be easily maintained and manipulated in the laboratory.[8] Another family of plant biologicals is Azotobacter and Phosphate Solubilizing Bacteria, which are non-symbiotic, free-living bacteria capable of fixing nutrients, producing phytohormones, and synthesizing antibiotics to protect plants from disease.[9,10] *Trachyspermum ammi* L is an Egyptian native herb that is cultivated in Iraq, Iran, Afghanistan, Pakistan, and India. Madhya Pradesh, Uttar Pradesh, Gujarat, Rajasthan, Maharashtra, Bihar, and West Bengal are among the states in India where it is grown. *T. ammi* L. is a highly regarded medicinally vital seed spice from the Apiaceae family. The fruits have excellent aphrodisiac effects, and the roots are diuretic. The fruits contain 2-4.4% ajwain oil, brown-colored oil. Thymol is the key ingredient in this oil, and it's used to treat gastrointestinal issues, a lack of appetite, and bronchial issues. On humans, the oil has fungicidal, antimicrobial, and anti-aggregatory properties. Ajwain is a traditional potential herb that is commonly used to treat a variety of human and animal illnesses. Flatulence, atonic dyspepsia, and diarrhoea can all be treated with it. Ajwain seed is bitter and pungent, and it has anthelmintic, carminative, laxative, and stomachic properties. It also treats stomach tumours, aches, and piles, as well as having anti-inflammatory and antioxidant properties. The essential oil in the fruits contains around 50% thymol, which is a powerful germicide, antispasmodic, and fungicide. In toothpaste and perfumes, thymol is also used.[11] We designed this study to investigate a plant biological assisted, environmentally friendly solution to boost *T. ammi* productivity, based on the beneficial effects of plant biologicals on plant productivity and the relevance of *T. ammi* for household and medicinal applications. Plant biologicals such as mycorrhizal fungi (*Sebacina vermifera*), nitrogen-fixing bacteria (*Azotobacter chroococcum*), and phosphate-solubilizing bacteria (*Pseudomonas fluorescens*) were used in this study to see how they affected *T. ammi* production. This is an attempt to establish mycorrhizal related connections between *Sebacina vermifera* and *T. ammi* while also comparing the effects of nitrogen-fixing bacteria (*A. chroococcum*) and phosphate solubilizing bacteria (*P. fluorescens*) on the same plant.

2. MATERIALS AND METHODS

2.1 Plant Material

During the rabi season, fruits of ajwain were collected from Punjab Agriculture University, Ludhiana, India. Fruits were treated for 5 minutes with 1% savlon (Johnson & Johnson, USA), then surface-sterilized for 1 minute with 70% (v/v) ethanol and washed three times with sterile double distilled water (SDDW). This was followed by a 2-minute treatment with 0.01% Bavistin (Saraswati Agro Chemicals Pvt. Ltd. India), 4-5 times SDDW rinses, and a 24-hour soak in distilled water. Soil samples from greenhouse and field pots were sent to College of Agriculture (Department of Soil Sciences), Punjab Agricultural University, Ludhiana (Ref. no. 1807/70) prior to testing, to determine the soil's chemical reserves. Chemical reserves of soil from both the pot and the field are enlisted in Table 1. The pot study was carried out in sterile soil at a greenhouse facility, where the soil was autoclaved in cotton bags for 1 hour at 121°C and then allowed to cool at room temperature before being repeated three times after 24 hours on three different days. Plants were cultivated in a greenhouse under regulated environmental conditions of 25±2°C, 16 hours of light/8 hours of darkness, 1,000 Lux light intensity, and 70% relative humidity. All of the field tests were carried out in farms and used a Randomized Complete Block Design (RCBD) (RCBD).[12,13]

Table 1: Chemical reserves of the soil

Sample	pH	Electrical conductivity (mmho/cm)	C (%)	P (kg/ha)	K (kg/ha)	Fe (kg/ha)	Zn (kg/ha)	Cu (kg/ha)	Mn (kg/ha)	N (%)
Pot	6.7	0.63	0.66	42.25	370.65	13.54	4.89	3.51	9.59	0.08
Field	7.4	0.59	0.48	32.37	311.35	12.26	4.10	1.53	7.36	0.06

Note: C-Carbon, P-Phosphorus, K-Potash, Fe-Ferrous, Zn-Zinc, Cu-Copper, Mn- Manganese, N-Nitrogen

2.2 Biofertilizers

The mycorrhizal fungi-*Sebacina vermifera* was procured from the Department of Biochemical Engineering and Biotechnology, Indian Institute of Technology, Delhi, India. *S. vermifera* cultures were kept on slants using a modified Kaefer-agar medium. The pH and temperature were kept at 6.5 and 30±1 °C, respectively. Slants were preserved at 4°C

after a 10-day incubation period.[12] The fungus was grown in 500 mL Erlenmeyer flasks with 100 mL Modified Kaefer liquid media using a gyratory shaker at 200 rpm at 30±1°C.[12,14,15] After an 8-day incubation period, the fungal culture (100 mL) was combined with 1 mL carboxy methyl cellulose (CMC). After that, 25 mL of CMC mixed culture was added to 75 g of sterilized talcum powder.[15] The preparation was kept in an airtight polythene bags at room temperature until it was used in the experiment. Ganesh Agro Service Centre, Moga, Punjab, India, provided commercially available biofertilizer preparations of Azotobacter (*Azotobacter chroococcum*) and Phosphate solubilizing bacteria (*P. fluorescens*).

2.2 Treatments

Control (CON), *A. chroococcum* (AZOTO), *P. fluorescens*-Phosphate solubilizing bacteria (PSB), and *S. vermifera* were the four treatment groups (SV). The CON group had a simple treatment without biologicals, whereas the AZOTO, PSB, and SV groups received a twofold treatment (soil and host fruits/plantlets) with varied schemes. Seed treatment (for emergence studies) in all groups except CON was done with a paste of 1 kg biomass in 1 L of water (enough for 10 kg of fruits). The paste was evenly applied to the seed surface, and the fruits were then dried in the shade before being planted in pots or the field. Plantlets were treated (for growth studies) by dipping roots in the same paste that was used for seed treatment and then sown immediately in pots or in the field. By inducing 1 kg biomass in 19 kg of vermicompost and mixing it with soil in a 60:40 ratio, the soil of pots was treated with AZOTO, PSB, and SV (soil: treated vermicompost). In contrast, treated vermicompost was dispersed as 150 kg treated vermicompost per hectare in the field. After 8 weeks, standing plants were treated (1 kg biomass in 19 kg vermicompost) using the drilling/drenching procedure.[12]

2.2 Germination Studies

Each treatment group's pot study was conducted in ten polythene pots with a capacity of 2 kg each (total 100 fruits for each treatment groups). 3/4thcapacity of the pot was filled with treated soil that had been soaked with water before the fruits were planted. After that, 10 healthy ajwain fruits were sown in individual pots with moistened treated soil. After that, the remaining 1/4thof the container was filled with treated dry soil, and all of the fruits were sown 2 cm below the soil surface in the pot. Finally, water was sprayed over the fruits to wet the dry soil. To avoid dryness, the soil was covered with straw and water was provided on a daily basis to avoid drought stress. The field research was conducted in an agricultural field where routine farming was carried out. Several ploughings and disking were used to make the soil permeable, allowing for healthy root development. The total trial area was 25×37 meters, with 24 plots of 4.95×4.95 meters each, each with well tilted and fine soil combined with a predetermined amount of vermicompost and treatments. A buffering zone of more than 1 meter separated each plot. Then 100 fruits from each group were sown in each plot, which had 10 rows and 10 columns, with each seed spaced 45 cm apart from the next seed. The soil layer was applied to the fruits in such a way that all fruits were covered 2 cm below the soil surface. A gentle spray of water was used to hydrate the soil. To avoid dryness, the soil was covered with straw, and water was provided on a daily basis to avoid drought stress. The germination study was carried out in both greenhouse and field circumstances until the germination was stable for 3 days and the following parameters were used to assess the results:

a) Germination percentage.[16]
b) Mean germination time.[17]
c) Germination index.[18]
d) T50 of germination.[19,20,21]
e) Seed vigour.[22]
f) Vigour index.[23]

2.2 Growth Studies

For the growth study, young and healthy 30 day old plantlets of identical size and growth stage were selected. Plant populations were maintained at 100 for each replicate of each treatment group in both greenhouse and field trials. Plantlets were sown in pots of 10 kg capacity but in the field, plantlets were sown in a plot consisting 10 rows and 10 columns and plantlets were interspaced at 45 cm to next plantlets. To avoid drought stress, both situations' soils were irrigated on a regular basis. Plants were uprooted periodically at an interval of 30 days *i.e.* 30, 60, 90 and 120 DAT (Day after transplantation) for growth evaluations. Evaluation was done using Total dry weight of the plant (root + shoot).

2.2 Phytochemical Studies

A sample of fruits was taken from each group's well-grown, healthy plants (120 days old) and dried at room temperature. In a blender, dried samples were ground. Hydro-distillation with a Clevenger device was used to extract powdered fruits for 4 hours. The oil samples were collected and stored in an airtight container at 4°C until further examination.

2.2 Gas Chromatography-Mass Spectrometry

Gas chromatography-mass spectrometry (GC-MS) was performed using a Thermo trace 1300 GC connected to a Thermo TSQ 8000 mass spectrometer using the electron impact ionization method. The temperature of the ion source was 230 degrees Celsius. The capillary column utilized was a TG-5MS (30 m ×0.25 mm, 0.25 µm film thickness). The temperature of the column was set to climb at a rate of 10 °C per minute from 50 °C (2 minutes) to 280°C. The temperature of the S/SL injector was kept at 250°C, and the injection volume was 1.0 µL. The temperature of the MS transfer line was kept at 280°C. Helium was used as the carrier gas, with a flow rate of 1 mL/min and a mass range of 50-500 m/z.[24]

2. Statistical Analysis

Except for GCMS, all tests were done in six replicates (sample size was six from each replicate). The information was presented in the form of a mean and standard deviation (SD). The data on emergence, EO yield, and the composition of distinct EO components were examined using oneway analysis of variance (ANOVA) and Tukey's post hoc test to compare means at the significance level of $p<0.05$. For growth study, two-way ANOVA was employed,

followed by a Bonferroni post-test for multiple comparison at the significance level of $p<0.05$. The GraphPad Prism v6 software suite was used for all statistical analyses.

3. RESULTS

3.1 Germination Studies

Emergence study on Ajwain was observed for 23 days under pot and field conditions. Of four groups, three groups viz. AZOTO, PSB and SV exerted significantly positive effects on emergence profile with respect to all the traits. During pot and field conditions, SV showed best results followed by PSB and AZOTO. Performance based data pertaining to individual treatment group is given in Table 5.12. Treatment with SV in pot conditions improved germination percentage, germination index, seedling vigour and vigour index by 2.15 (84%), 2.45 (6.03), 1.29 (107.5 mm) and 2.78 (9030) times respectively, whereas PSB improved same traits by 1.95 (76%), 2.16 (5.32), 1.04 (86.6 mm) and 2.03 (6581.6) times respectively. Similarly, AZOTO improved the same traits by 1.69 (66%), 1.76 (4.33), 1.02 (85 mm) and 1.73 (5610), when compared to CON (39%, 2.46, 83.30 mm and 3248.7 respectively). On the other hand, mean germination time was reduced to 0.88 (14.31 days) and 0.89 (14.53 days) and 0.96 (15.61) times respectively for SV, PSB and AZOTO, when compared with CON (16.33 days), PSB reduced T_{50} germination by 0.91 (13.82 days) times, whereas AZOTO and SV exceptionally increased the T_{50} by 1.10 (16.28 days) and 1.07 (16.71 days) times respectively when compared to CON (15.25 days) (Table 2). Results of all three treatments (SV, PSB and AZOTO) during field study were same as the effects observed during pot studies (except T_{50}). Here, germination percentage, germination index, seedling vigour and vigour index for SV were improved by 1.59 (51%), 1.89 (3.49), 1.36 (100 mm) and 2.17 (5100) times respectively, with PSB, these traits were improved by 1.44 (46%), 1.56 (2.88), 1.17 (85.5 mm) and 1.68 (3933) times respectively, similarly with AZOTO these traits were improved by 1.09 (35%), 1.15 (2.13), 1.06 (77.5 mm) and 1.16 (2712.5) times respectively, when compared to CON (32%, 1.85, 73.3 mm and 2345.6 respectively). On the other hand, mean germination time was reduced by 0.86 (15.12 days), 0.93 times (16.43 days) and 0.95 (16.77 days) for SV, PSB and AZOTO respectively, when compared to CON (17.66 days). Similarly, T_{50} germination was reduced by 0.93 (15.71 days), 0.98 (16.43 days) and 0.99 (16.64) times for SV, PSB and AZOTO respectively, when compared to CON (16.81 days) (Table 2).

Table 2: Effect of tested biofertilizers on emergence of Ajwain (*Trachyspermum ammi*).

Parameters	POTS				FIELDS			
	CON (Mean±SD)	AZOTO (Mean±SD)	PSB (Mean±SD)	SV (Mean±SD)	CON (Mean±SD)	AZOTO (Mean±SD)	PSB (Mean±SD)	SV (Mean±SD)
Germinationpercentage (%)	39.00±2.00	66.00±1.53^{a}	76.00±2.00^{a,b}	84.00±1.53^{a,b,c}	32.00±4.51	35.00±3.00	46.00±2.52^{a,b}	51.00±3.00^{a,b}
Mean germination time (Days)	16.33±0.93	15.61±0.57	14.53±0.36^{a}	14.31±0.99^{a,b}	17.66±0.62	16.77±0.66	16.43±0.88^{a}	15.12±0.80^{a,b,c}
Germination index	2.46±0.42	4.33±0.40^{a}	5.32±0.28^{a,b}	6.03±0.20^{a,b,c}	1.85±0.40	2.13±0.30^{a}	2.88±0.36^{a,b}	3.49±0.46^{a,b}
T_{50} germination (Days)	15.25±0.76	16.71±0.51^{a}	13.82±0.50^{a,b}	16.28±0.93^{c}	16.81±1.65	16.64±1.10	16.43±1.46	15.71±1.30
Seedling vigour (mm)	83.30±2.50	85.00±3.51	86.60±2.50	107.50±2.50^{a,b,c}	73.30±2.62	77.50±1.91^{a}	85.50±2.57^{a,b}	100.00±2.00^{a,b,c}
Vigour index	3248.70±264.60	5610.00±357.51^{a}	6581.60±363.00^{a,b}	9030.00±373.98^{a,b,c}	2345.60±418.27	2712.50±297.84	3933.00±328.43^{a,b}	5100.00±402.01^{a,b,c}

Note: Emergence response of fruits to various treatments in pots and field trials: data expressed as mean ± SD of six replicates. Superscripts with different letters (a-c) within the same row represent significance level as $p^{a}<0.05$ v/s CON; $p^{b}<0.05$ v/s AZOTO; $p^{c}<0.05$ v/s PSB; CON- Normal control; AZOTO- Azotobacter; PSB- Phosphate solubilizing bacteria; SV- S. vermifera.

3.2 Growth Studies

Under both (pot and field) conditions, although, certain enhancements in growth parameters were recorded by treatments in different patterns but effects of SV were significantly higher at each growth stages (Fig. 1). At final growth stage i.e. 120 DAT, most prominent effect on cumulative total dry weight of plant was recorded with SV. Under pot conditions, the enhancements were 41.5%, 24.05% and 14.16%, whereas, under field conditions the enhancements were 37.79%, 30.41% and 37.79% in comparison to CON, AZOTO and PSB respectively (Fig. 2). These enhancements patterns were not restricted to particular system of plant but found to be almost equal in shoot system and root system. Similar enhancement as recorded on all other parameters is tabulated in Table 3.

Table 3: Effect of tested biofertilizers on growth of Ajwain (*Trachyspermum ammi*) at 120 DAT.

Parameters	Pots				Field			
	CON (Mean±SD)	AZOTO (Mean±SD)	PSB (Mean±SD)	SV (Mean±SD)	CON (Mean±SD)	AZOTO (Mean±SD)	PSB (Mean±SD)	SV (Mean±SD)
Length of the plant (cm)	69.40±2.65	84.64±2.68^{a}	100.24±2.71^{a,b}	119.83±2.73^{a,b,c}	64.12±2.35	75.72±2.37^{a}	82.13±2.38^{a,b}	103.20±2.41^{a,b,c}
Shoot length (cm)	52.17±3.20	64.00±3.28^{a}	77.80±3.24^{a,b}	91.50±3.23^{a,b,c}	49.37±2.40	59.20±2.42^{a}	65.00±2.44^{a,b}	83.17±2.46^{a,b,c}
Root length (cm)	17.23±0.61	20.64±0.63^{a}	22.44±0.66^{a,b}	28.33±0.67^{a,b,c}	14.75±0.76	16.52±0.77^{a}	17.13±0.78^{a}	20.03±0.79^{a,b,c}
Shoot branching (No.)	14.32±0.35	15.73±0.34^{a}	16.87±0.32^{a,b}	23.85±0.31^{a,b,c}	11.84±0.46	12.29±0.44	12.97±0.47^{a,b}	16.63±0.45^{a,b,c}
Root branching (No.)	24.21±0.50	26.24±0.53^{a}	27.61±0.54^{a,b}	32.65±0.57^{a,b,c}	23.45±0.49	25.28±0.51^{a}	26.45±0.52^{a,b}	31.39±0.54^{a,b,c}
Major root length (cm)	16.23±0.54	20.10±0.56^{a}	21.05±0.58^{a,b}	27.20±0.61^{a,b,c}	13.90±0.73	15.67±0.74^{a}	16.28±0.76^{a}	19.18±0.77^{a,b,c}
Minor root length (cm)	225.67±9.83	248.00±9.76^{a}	263.00±9.30^{a,b}	279.50±9.20^{a,b,c}	218.57±8.98	238.24±9.05^{a}	256.65±9.07^{a,b}	270.42±9.12^{a,b,c}
Total fresh weight (g)	34.89±0.79	38.15±0.82^{a}	41.82±0.81^{a,b}	51.05±0.83^{a,b,c}	34.20±0.76	35.99±0.78^{a}	39.25±0.81^{a,b}	48.56±0.82^{a,b,c}
Shoot fresh weight (g)	26.05±0.67	28.23±0.69^{a}	30.21±0.71^{a,b}	37.79±0.72^{a,b,c}	25.65±0.67	26.99±0.69^{a}	29.44±0.68^{a,b}	36.42±0.66^{a,b,c}
Root fresh weight (g)	8.84±0.15	9.92±0.16^{a}	11.61±0.13^{a,b}	13.26±0.14^{a,b,c}	8.55±0.13	9.00±0.15^{a}	9.81±0.16^{a,b}	12.14±0.17^{a,b,c}
Shoot dry weight (g)	9.32±0.23	10.71±0.24^{a}	11.33±0.26^{a,b}	12.93±0.27^{a,b,c}	7.92±0.30	8.38±0.32^{a}	9.19±0.31^{a,b}	10.79±0.34^{a,b,c}
Root dry weight (g)	3.27±0.05	3.66±0.06^{a}	4.29±0.06^{a,b}	4.90±0.05^{a,b,c}	2.84±0.08	2.99±0.07^{a}	3.26±0.08^{a,b}	4.04±0.09^{a,b,c}
Total dry weight (g)	12.59±0.36	14.37±0.39^{a}	15.62±0.32^{a,b}	17.83±0.45^{a,b,c}	10.76±0.36	11.37±0.36^{a}	12.45±0.37^{a,b}	14.83±0.38^{a,b,c}

Growth response of plantlets to various treatments in pots and field trials: data expressed as mean ± SD of six replicates. Superscripts with different letters (a-c) within the same row represent significance level as p^{a}<0.05 v/s CON; p^{b}<0.05 v/s AZOTO; p^{c}<0.05 v/s PSB; CON- Normal control; AZOTO- Azotobacter; PSB- Phosphate solubilizing bacteria; SV- S. vermifera.

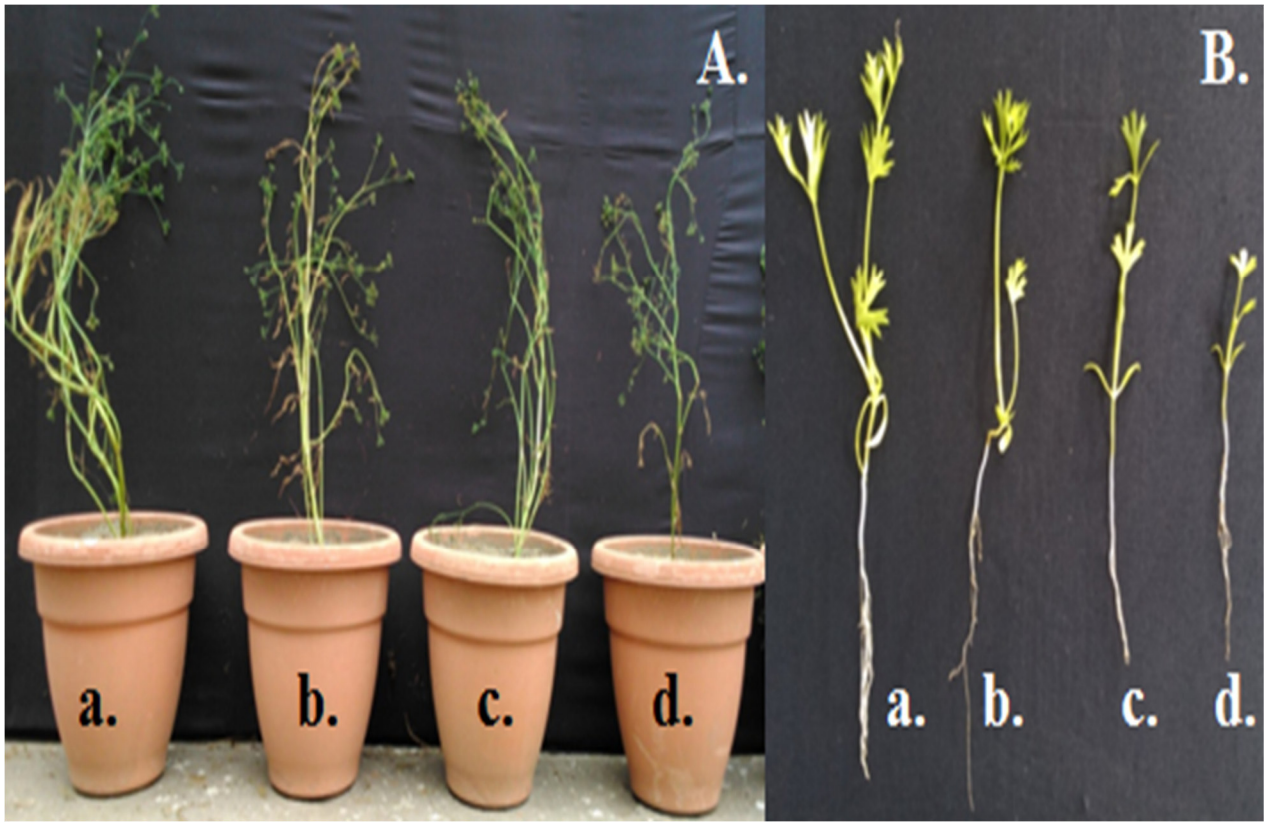

Fig 1: (A.) Plants under pot trials at 120 DAT (B.) Plants under field trials at 60 DAT; (a) *S. vermifera* (b) Phosphate solubilizing bacteria (c) Azotobacter (d) Control.

However, when relative growth rates (for total dry weight) were compared, only SV showed highest growth rate when compared with CON and other experiments (Fig 3). Growth rates (total dry weight) of AZOTO treated plants were found to be very moderate to CON experiments. At 120 DAT, growth rates of AZOTO and PSB were same as CON experiment under both pot and field conditions. It was also observed that growth rate of plant under field conditions were slightly low in comparison to the pot condition and growth rate of AZOTO and PSB were almost same at field conditions. In both conditions (pot and field), increasing growth rates were observed during from 30 DAT-90 DAT. In all cases, the maximum growth rate was observed at 90 DAT and growth rate was declined after 90 DAT days (Fig. 3)

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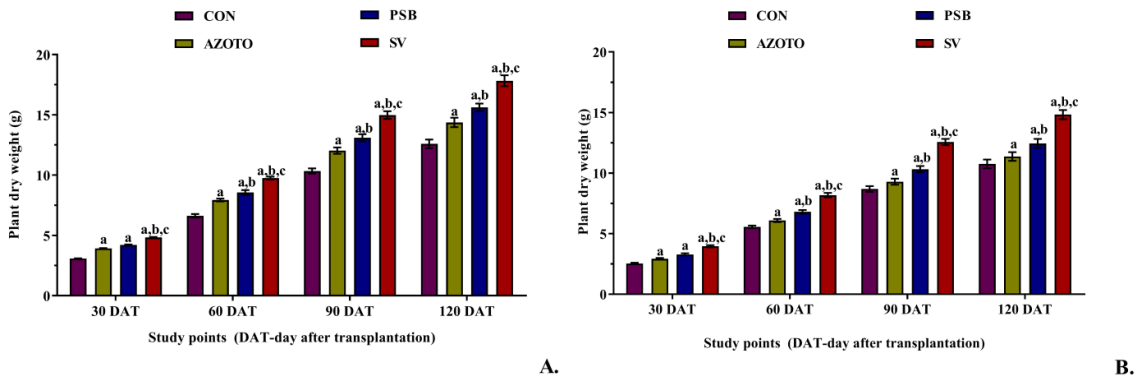

Fig 2: Effect of treatments on (A.) total dry weight in pot; (B.) total dry weight in field; data expressed as mean ± SD of six replicates. [Superscripts with different letters (a-c) within the same harvesting group represent significance level as p^a<0.05 v/s CON; p^b<0.05 v/s AZOTO; p^c<0.05 v/s PSB; CON- Normal control; AZOTO- *Azotobacter*; PSB- Phosphate solubilizing bacteria; SV- *S. vermifera*].

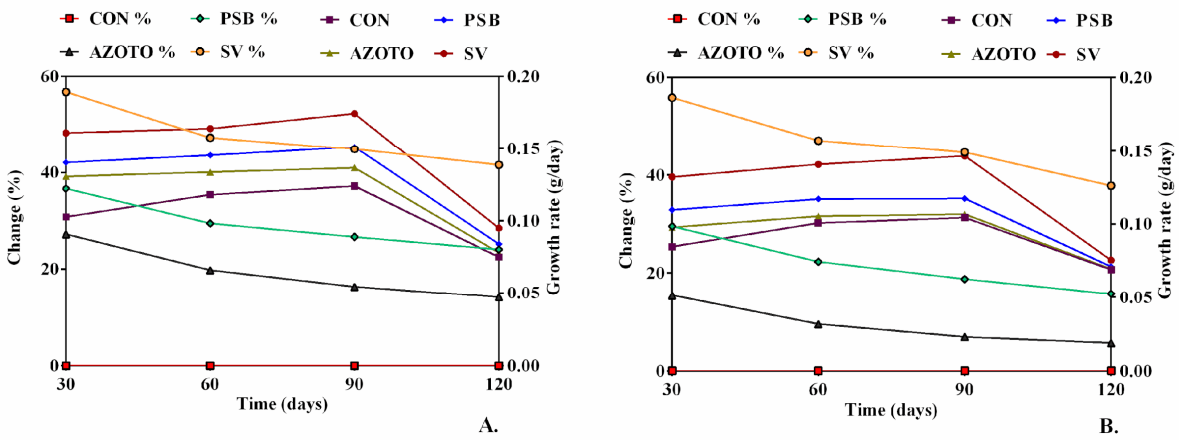

Fig 3: Effect of treatments on relative growth rate (A.) total dry weight in pot; (B.) total dry weight in field; data expressed as growth rate for dry weight as g/day.

The elemental analysis (CHN) of air dried matter of plant revealed that SV facilitated the highest nutrient uptake and overall balanced accumulation in significant manner (Table 4). SV treated plants were recorded with highest accumulation of nutrients in pots 35%, 20.87% and 22%, whereas in field 28%, 15.5% and 23% for nitrogen, carbon and hydrogen respectively when compare to CON.

Table 4: Effect of tested biofertilizers on elemental accumulation by Ajwain (*Trachyspermum ammi*) at 120 DAT.

Elements	Pot				Field			
	CON (Mean±SD)	AZOTO (Mean±SD)	PSB (Mean±SD)	SV (Mean±SD)	CON (Mean±SD)	AZOTO (Mean±SD)	PSB (Mean±SD)	SV (Mean±SD)
Nitrogen	2.11±0.09	2.19±0.08	2.14±0.06	$2.85\pm0.07^{a,b,c}$	2.03±0.07	2.10±0.06	2.06±0.05	$2.60\pm0.08^{a,b,c}$
Carbon	38.33±1.11	45.10 ± 1.12^{a}	38.36 ± 1.18^{b}	$46.33\pm1.16^{a,c}$	37.91±1.14	43.70 ± 1.17^{a}	37.80 ± 1.16^{b}	$43.80\pm1.12^{a,c}$
Hydrogen	5.59±0.16	6.68 ± 0.15^{a}	5.42 ± 0.14^{b}	$6.84\pm0.21^{a,c}$	5.32±0.16	6.24 ± 0.18^{a}	5.21 ± 0.15^{b}	$6.56\pm0.14^{a,b,c}$

Growth response in essential elements accumulation to various treatments in pots and field trials: data expressed as mean ± SD of six replicates. Superscripts with different letters (a-c) within the same row represent significance level as p^a<0.05 v/s CON; p^b<0.05 v/s AZOTO; p^c<0.05 v/s PSB; CON- Normal control; AZOTO- Azotobacter; PSB- Phosphate solubilizing bacteria; SV- S. vermifera.

3.3 Phytochemical Studies

3.3.1 Yield of Essential Oils

Application of microbe containing bioformulations was recorded with a marked increase in growth and yield of EO in Ajwain. Hydro distillation of air dried fruits of ajwain revealed that, although all treatments enhanced the EO accumulation to a certain level but the enhancements were significantly higher with SV in comparison to CON, AZOTO and PSB. It is worthy to mention that SV enhanced the yield of EO by 100% (pot) and 53.8% (field), whereas, the enhancement was 77.8% in comparison to AZOTO and PSB under pot conditions, 33% and 25% in comparison to AZOTO and PSB under field condition. Here, AZOTO and PSB equally enhanced yield of EO by 12.5% in pot as well as 15% by AZOTO and 23% by PSB in field condition (Fig. 4).

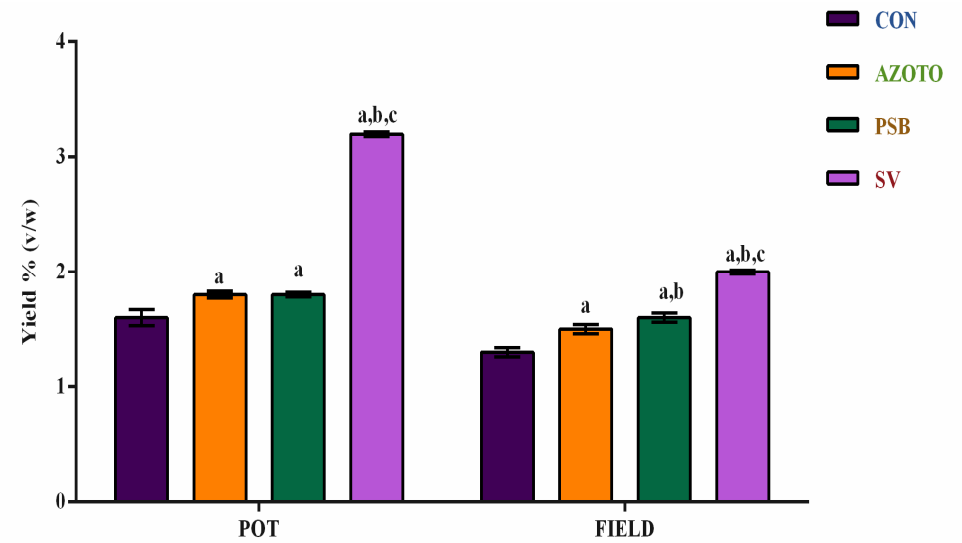

Fig 4: Effect of treatments on yield of EO of plant fruits under pot and field conditions: data expressed as mean ± SD of six replicates. [Superscripts with different letters (a-c) within the same condition group represent significance level as p^a<0.05 v/s CON; p^b<0.05 v/s AZOTO; p^c<0.05 v/s PSB; CON-Normal control; AZOTO- *Azotobacter*; PSB-Phosphate solubilizing bacteria; SV-*S. vermifera*].

3.3.2 GC-MS Analysis

Analysis of extracted EO revealed the presence of 13 identified components (Table 5). Each treatment was recorded with a specific augmentation towards the major as well as trace components of EO (Fig.5-8). Major altered components were thymol, p-cymene and ϒ-terpinene. Treatment of SV was associated with marked increase in thymol (50.5%) of whole content of EO, while drastic decrease in p-cymene (17%) and ϒ-terpinene (16%) of whole content of EO (both in pot and field) was recorded. Contrary to this CON group showed a significant lower content of thymol (pot-12.9% and field-11.9%), while higher amount of p-cymene (pot-36.7% and field- 34%) and ϒ-terpinene (pot-33.7% and field-31.7%). Similar to CON, AZOTO exhibited the lower level of thymol (pot-17.5% and field-14%) and higher level of p-cymene (pot-39% and field-37%) and ϒ-terpinene (pot-32% and field-30%). Among four treatment groups only PSB group exhibited a moderate effect on all three major constituents, thymol, p-cymene and ϒ-terpinene as 23.6%, 24.6% and 24% in pots and 23.6%, 22.8% and 23.9% respectively in field experiments (Fig. 9).

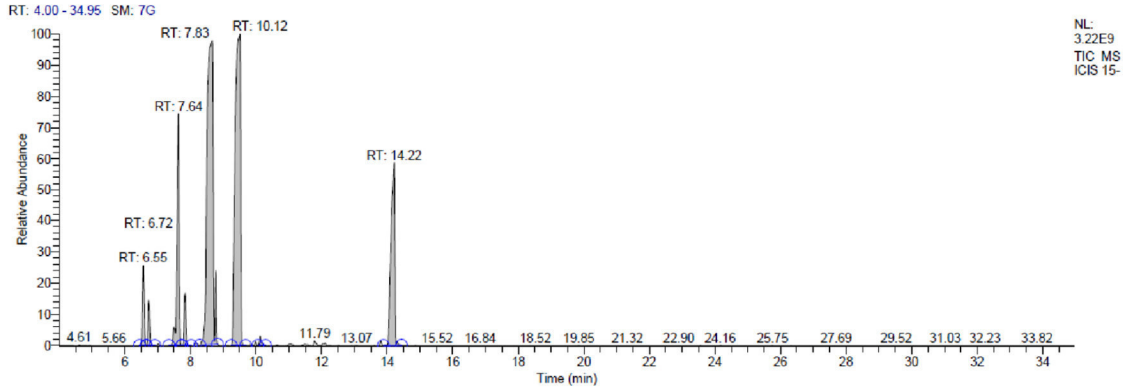

Fig 5: Representative chromatogram of control group.

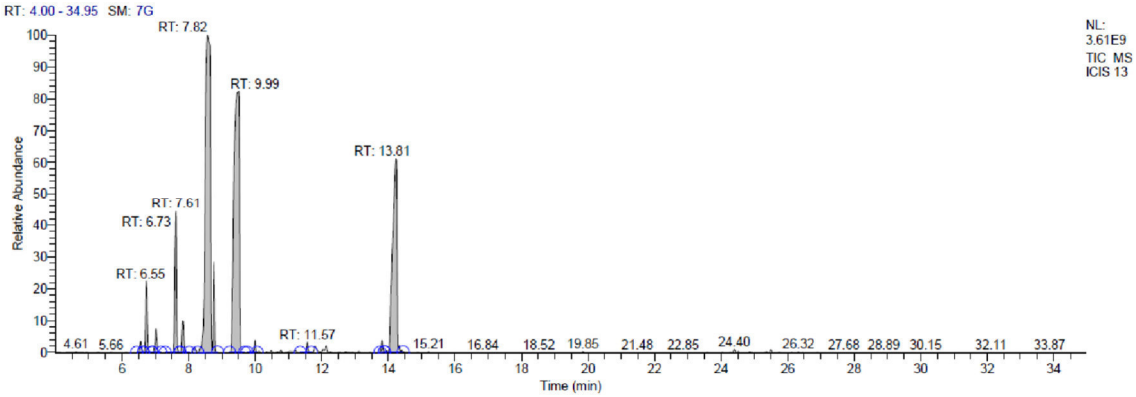

Fig 6: Representative chromatogram of Azotobacter group.

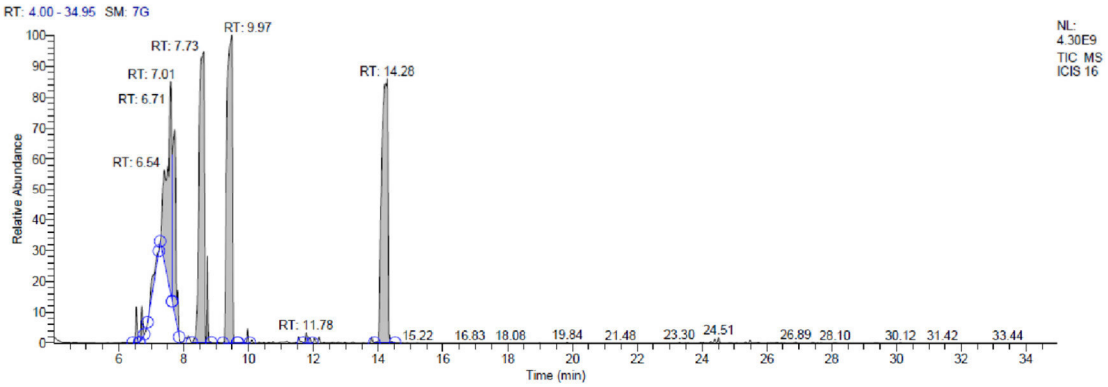

Fig 7: Representative chromatogram of Phosphate solubilizing bacteria group.

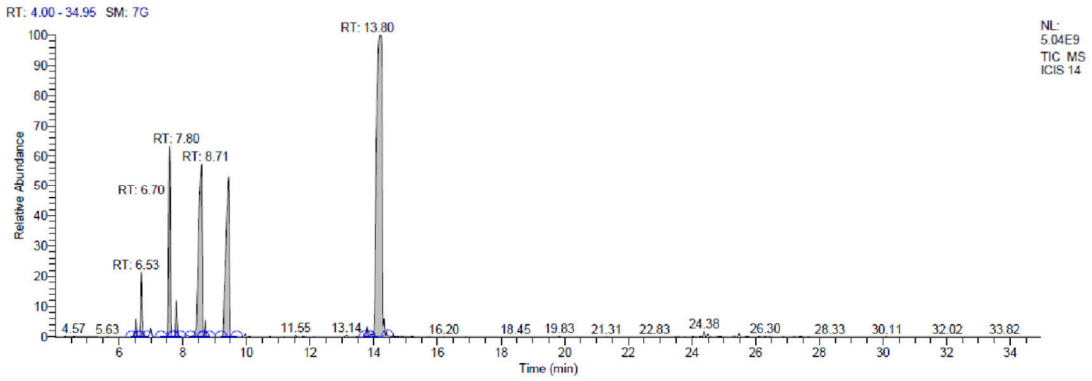

Fig 8: Representative chromatogram of *Sebacina vermifera* group.

Table 5: Effect of tested biofertilizers on EO components of Ajwain (*Trachyspermum ammi*).

S.No.	RT	Components	Pot				Field			
			CON (Mean±SD)	AZOTO (Mean±SD)	PSB (Mean±SD)	SV (Mean±SD)	CON (Mean±SD)	AZOTO (Mean±SD)	PSB (Mean±SD)	SV (Mean±SD)
1.	6.55	α-thujene	2.93±0.68	0.30±0.10^{a}	0.75±0.07^{a}	0.75±0.02^{a}	3.80±0.09	3.28±0.12^{a}	2.73±0.08^{a,b}	1.22±0.04^{a,b,c}
2.	6.73	α -Pinene	1.40±0.08	2.40±0.16^{a}	0.71±0.03^{a,b}	2.73±0.11^{a,b,c}	2.20±0.07	2.38±0.14^{a}	1.69±0.02^{a,b}	2.76±0.12^{a,b,c}
3.	7.02	Camphene	-	1.02±0.06	1.08±0.04			0.99±0.05	0.98±0.04	
4.	7.61	β-pinene	10.00±0.10	4.70±0.59^{a}	15.68±1.48^{a,b}	9.67±0.56^{b,c}	9.99±0.10	7.62±0.54^{a}	14.88±0.62^{a,b}	9.00±0.57^{a,b,c}
5.	7.82	α -Myrcene	1.89±0.04	1.26±0.11^{a}	8.65±0.39^{a,b}	1.65±0.04^{b,c}	3.82±0.03	2.15±0.04^{a}	8.63±0.37^{a,b}	1.65±0.03^{a,b,c}
6.	8.57	p-Cymene	36.72±1.64	39.37±3.59	24.60±0.70^{a,b}	17.33±0.55^{a,b,c}	34.27±0.98	37.36±0.99^{a}	22.81±0.96^{a,b}	17.66±0.98^{a,b,c}
7.	8.71	D-Camphene	-	-	-	0.55±0.04	-	-	-	0.55±0.03
8.	9.50	ϒ-Terpinene	33.77±2.28	32.32±2.84^{a}	24.12±0.72^{a,b}	16.42±0.25^{b,c}	31.70±0.99	30.24±0.97	23.91±0.99^{a,b}	16.27±0.97^{a,b,c}
9.	9.99	Terpinolene	-	0.36±0.02	0.27±0.02	-	-	0.85±0.02	0.29±0.02	-
10.	10.12	*cis*-sabinene hydrate	0.35±0.03	-	-	-	2.32±0.03	-	-	-
11.	11.57	Borneol	-	0.37±0.02	0.48±0.03	-	-	0.34±0.02	0.46±0.03	-
12.	13.81	Anethole	-	0.36±0.03	-	0.37±0.03	-	0.34±0.03	-	0.37±0.03
13.	14.23	Thymol	12.92±0.58	17.54±1.01^{a}	23.65±1.04^{a,b}	50.52±1.02^{a,b,c}	11.90±0.49	14.44±0.99^{a}	23.62±0.96^{a,b}	50.52±0.95^{a,b,c}

Response of various treatments on EO components in pots and field trials: data expressed as mean ± SD of three replicates. Superscripts with different letters (a-c) within the same row represent significance level as p^{a}<0.05 v/s CON; p^{b}<0.05 v/s AZOTO; p^{c}<0.05 v/s PSB; CON- Normal control; AZOTO- Azotobacter; PSB- Phosphate solubilizing bacteria; SV- S. vermifera.

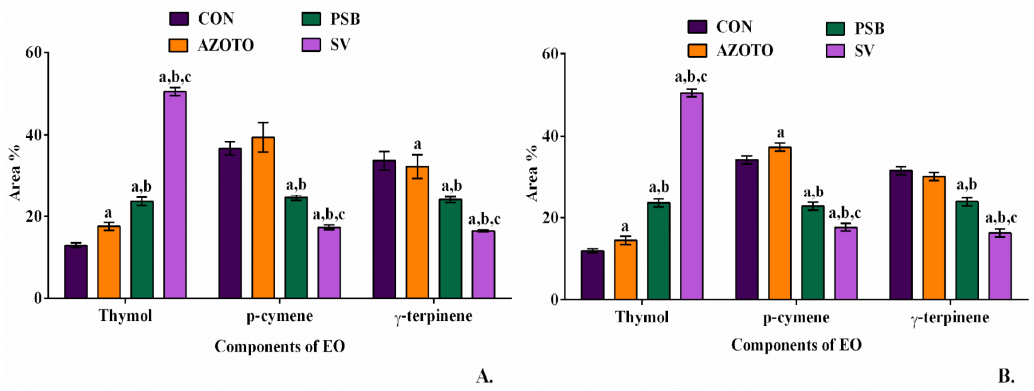

Fig 9: Effect of treatments on EO components (A.) in pot; (B.) in field; data expressed as mean ± SD of three replicates. [Superscripts with different letters (a-c) within the same components group represent significance level as p^a<0.05 v/s CON; p^b<0.05 v/s AZOTO; p^c<0.05 v/s PSB; CON- Normal control; AZOTO- *Azotobacter*; PSB- Phosphate solubilizing bacteria; SV- *S. vermifera*].

4. DISCUSSION

The active symbiotic relationship between *S. vermifera* and *Trachyspermum ammi* was discovered in this study. Previous research has shown that *S. vermifera* can successfully establish a relationship with a wide range of plant species, including *Trigonella Foenumgraecum*[12], *Foeniculum vulgare*[13], *Coriandrum sativum*[25], *Anethum graveolens* L.[26], *Nicotiana attenuate*[27], *Thymus vulgaris*[28], *Brassicaceae plants*[29], *Panicum virgatum*[30,31] and *Oryza sativa*.[32] All of the microorganisms that were studied had a beneficial impact on germination properties. *A. chroococcum* had a comparable beneficial effect on the germination of *Dodonaea viscoseseeds*[33], and *P. fluorescens* had a positive effect on the germination of *Vicia faba*[34]. Although the underlying mechanisms for selected microbes' stimulatory effects is still unknown, it is thought that the enhanced germination is the result of phytohormones and other complex mixtures of biologically active compounds and growth-promoting metabolites modulated by *A. chroococcum*[35,36,37]and *P. fluorescens*[38,39]. Similarly, fungal inoculant (*S. vermifera*) was found to have a favourable effect on Ajwain seed germination. Previous research on *Coriandrum sativum*[12], *Foeniculum vulgare*[13], *N. attenuate*, *P. virgatum*, and *Cynorkis purpurea*[40,41] supports these findings. It's possible that the performance of *S. vermifera* is influenced by planting species, application processes, experimental designs, and the conditions under which trials are conducted. Maighal *et al.* (2016)[42] confirm our findings and indicate that two factors, "mycorrhiza" and "seed species," are responsible for seed viability and germination. The growth-promoting characteristics of examined microorganisms are represented by morphological evaluation. *A. chroococcum*[43,44], *P. fluorescens*[45,46] and *S. vermifera*[32,40] have all previously been observed in the same way. Dolatabadi *et al.* (2011)[28] also found that fungal infected *T. vulgaris* had significantly increased fresh and dry weight. Plant length, fresh weight, dry weight, root system, and number of nodes have all been found to be increased in *S. vermifera* colonized plants.[29,47] Previous research suggested that AMF inoculation caused enhanced plant growth due to early expression of developmentally regulated genes[48]and phytohormonal involvements.[49] Eventually, the phytohormonal modulator effects of *A. chroococcum*, Pseudomonas species, and AM-like fungi (*P. indica*, which is closely related to *S. vermifera*) have been demonstrated.[49-53] Furthermore, better nutrient uptakes, particularly nitrogen and phosphorus, may be attributed to improved plant development. Plant biomass allocation, accumulation, and conspicuous growth, as well as an excellent seed yield and EO production, are significantly influenced by nitrogen and phosphorus.[54]It is well known that *A. chroococcum* fixes nitrogen to soil[55,56] and the Pseudomonas genera are well known as phosphate solubilizing bacteria, which fixes non-utilizable phosphate to utilizable forms for plants[57], whereas, *S. vermifera* is not limited to a single nutrient but has the ability to fix and efflux a wide range of macro- and micronutrients in addition to nitrogen and phosphorus[40], *S. vermifera* has, in fact, contributed more than other treatments to the exploration of huge soil volumes, increased nutrient intake, and mass accumulation. Increased nutrient intake has contributed to bulk accumulation, according to elemental analysis of CHN from a dry matter of plants. Microbe inoculation also changed the yield of EO, resulting in a higher quantity and quality of components. *A. chroococcum* increases the synthesis of EO in *Cymbopogon martini* and *F. vulgare*[58,59] according to a comparable study. *P. fluorescens* has also been studied for its ability to enhance EO in *Ocimum basilicum* and *Origanum majorana.*[60,61] *S. vermifera* considerably boosted the yield of EO in *Coriandrum sativum*[25], *Foeniculum vulgare*[13], *Anethum graveolens* L.[26] and *T. vulgaris*[29], according to previous research. Although the underlying mechanism for the increase in EO is unknown, it could be due to defensive responses[61], morphological features[62,63], up-regulation of biosynthetic genes[63-65] and increased P availability.[66-70] *S. vermifera* may have been related with the aforementioned parameters, and indeed, higher N and P efflux may have encouraged isopentenyl pyrophosphate (IPP) and dimethylallyl pyrophosphate (DMAPP) production better than other microorganisms, resulting in higher EO yield in treated plants. Some investigations have shown that *A. chroococcum* increased the concentration of Anethole in *F. vulgare*[59], which is a significant ingredient of EO. In *Linum album*, *S. vermifera* increased synthesis of podophyllotoxin and its 6-methoxy derivative. Additionally, fungal precursors and elicitors are commonly used to boost secondary metabolites in plant cell culture.[71-75]Artemisinin and withaferin-A, two economically important secondary metabolites, have already been successfully enhanced using a cell culture approach aided by a fungal elicitor.[76,77]Although the underlying mechanism(s) for the increase in main EO components is unknown, it is thought that activation of biosynthetic genes, modification of phytohormones, improved physiology, and a larger input of N and P may enhance the particular class of secondary metabolites. It additional stimulus generated by SV for the expression of CYP71D178 was strong enough to reach the metabolic reaction up to final stage along with the overall balanced nutrient availability might have served as ideal substrate for the production of final product thymol. The expression of gene is highly supported by Ormeno and Fernandez (2012)[78] that a higher expression of early step or late step genes leads to higher production of thymol in *Thymus vulgaris*. Further, enhancement in plant biomass considered as the second great reason for monoterpene biosynthesis because of availability of ideal substrate.[79]A possible biosynthetic pathway for the enhancement of particular component upon treatment with microbe-based biofertilizers is given in Fig.10.

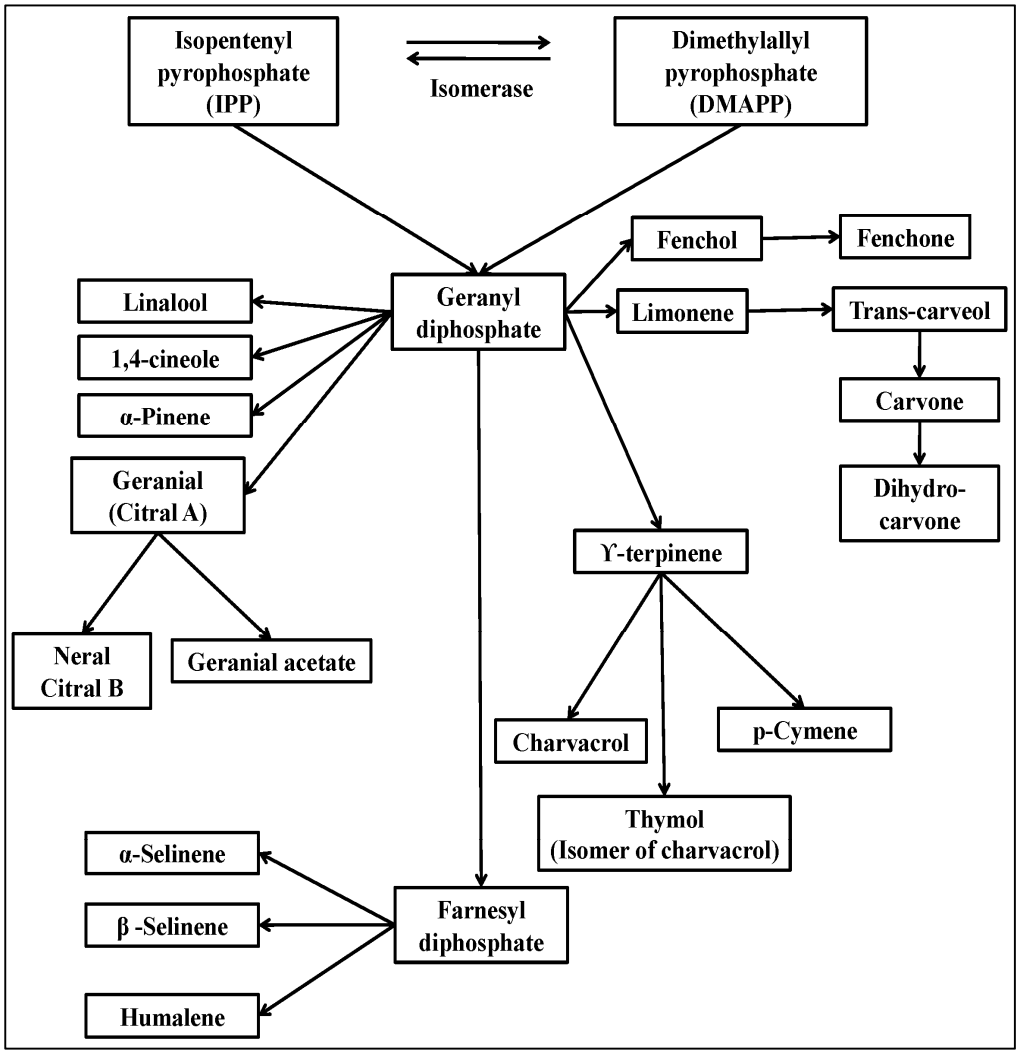

Fig 10: A possible biosynthetic pathway for the enhancement of a particular component upon treatment with microbe-based biofertilizers.

Baldi et al. 2008 confirmed this idea by stating that S. vermifera could increase secondary metabolite production by activating defense mechanisms. Baldi et al. (2010)[8] also showed that a fungal elicitor could stimulate the biosynthetic pathway, resulting in increased secondary metabolites in cell culture; this was further supported by phenylalanine ammonia-lyase (PAL) activity, which is a short limiting step in the synthesis of secondary lignin metabolites.[80,81]

5. CONCLUSION

Although AZOTO and PSB could contribute to improved ajwain plant performance, *S. vermifera* provided a greater magnitude of multifaceted benefits for the Ajwain plant in all aspects and supported emergence, plant growth, stimulated defensive mechanisms, maintained plant health and vitality, and increased EO yield and thus secondary metabolite yield. We suggest *S. vermifera* as a potential alternative to achieving eco-friendly and sustainable farming methods in general and in particular to ajwain, by extrapolating the overall results and assessing the efficacy.

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8. CONFLICTS OF INTEREST

The authors declare that they have no conflicts of interest.

9. AUTHORS CONTRIBUTION STATEMENT

The corresponding author, Dr. Ashish Baldi designed the work, supervised the experimentation, reviewed and edited the manuscript. Mr. Kamal Jeet performed the experiment, collected data, analyzed and prepared the manuscript.

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