Indian Journal of Ecology (2022) 49(3) (SI): 1208-1213
DOI: https://doi.org/10.55362/IJE/2022/3646

Manuscript Number: 3646
NAAS Rating: 5.79

Effect of Microbial Bio-Elicitors on Yield and Chemical Composition of Essential Oil in *Pelargonium graveolens* L.

Shivani Negi, Abdul Mazeed, Pooja Maurya, Dipender Kumar[1] and Priyanka Suryavanshi*

Division of Crop Production and Protection
CSIR-Central Institute of Medicinal and Aromatic Plants, Lucknow-226 015, India
[1]CSIR-Central Institute of Medicinal and Aromatic Plants, Research Centre, Pantnagar-263 149, India
**E-mail: priyanka@cimap.res.in*

***Abstract*:** The effect of arbuscular mycorrhiza fungi (AMF) and plant growth-promoting bacteria (PGPB) namely *Azotobacter*, *Advenella spp*, NPK liquid consortia (*Azotobacter, Azospirillum,* phosphate solubilizers and potassium solubilizers), and zinc solubilizing bacteria on rose scented geranium was evaluated under pot culture study. The microbial bio-elicitors significantly influenced all growth related parameters, aerial and root biomass, soil fertility status, microbial enzymes, oil yield and quality. Dual inoculation of AMF with *Advenella spp* has resulted in the highest synergistic effect as evidenced by the highest crop growth indices like plant height, number of branches and enhanced dry herb biomass and oil yield significantly higher than non-inoculated control. GC-MS studies showed that citronellol (23.15%-32.56%), geranial (8.48%-12.85%), citronellyl formate (5.94%-9.89%), isomenthon (6.16%-7.96%) and 10-epi-Y-Eudesmol (3.72%-6.98%) were the major components.

***Keywords*:** *Pelargonium graveolens*, AMF, PGPB, Essential oil, Chemical composition

Rose scented geranium *(Pelargonium graveolens* L.) (Geraniaceae) is a commercially important perennial aromatic plant (Fekri et al 2021). This plant species is commercially cultivated mainly in Algeria, Egypt, Morocco, India and China (Verma et al 2016). It is among the top 20 essential oils produced worldwide, with important applications in aromatherapy and pharmaceutical corporations (Rao 2002). Rose scented geranium has the medicinal property to cure dysentery, diarrhoea and colic disorders (Shawl et al 2006) and menorrhagia (Verma et al 2014). Presently, India requires about 200 tonnes of geranium oil annually but invests a lot of foreign currency in its import as its domestic production is only about 20 tonnes (Nilofer et al 2018). Therefore, there is a need to enhance both yield and essential oil quality in case of rose scented geranium, but through low cost eco-friendly technologies. Microbial bio-elicitors improves the growth rate of plants and soil health as act as plant strength, phyto-stimulator, plant health improvement and have the potential to improve soil fertility status (Babalola 2014). The most widely used biofertilizers which contribute nitrogen, phosphorus, and potassium to plants are *Azotobacter, Azospirillum* and PSB. Rhizospheric soil of several species of medicinal plants has a symbiotic association with AMF (Assis et al 2020). They play a crucial role in mineralization and cycling of nutrients in plants (Jansa et al 2019), reducing the chemical fertilizer dose by 25-50% (Rana et al 2012). The decrease in input cost of geranium by adopting eco-friendly nutrient management practices particularly through using microbial inoculants for increasing bioavailability of nitrogen, phosphorus and potash can be a viable option. The present investigation was carried out to evaluate the role of dual inoculation of AMF and PGPR inoculants as microbial bio-elicitors on enhancing yield and chemical composition of rose scented geranium.

MATERIAL AND METHODS

Experimental site: The experiment was conducted at CSIR–Central Institute of Medicinal and Aromatic Plants (CIMAP), which is situated at 26°5´ N latitude and 80°5´ E longitude with an altitude of about 120 m above MSL at Lucknow, Uttar Pradesh, India. The site's climate is defined by scorching summers, relatively cold winters and an average annual precipitation of 1000 mm. The soil in the experimental field was alkaline having a pH of 8.03 and classified as loamy sand with N, P, and K content of 202.10, 52.06, and 152.24 kg ha^{-1}respectively and soil organic carbon content of 3.21 g kg^{-1}soil.

Experimental details: Randomized complete block design (RCBD) pot experiment with four replicates was set up. In an earthen pot with a diameter of 20 cm and a capacity of ten litters, nine kg of air dried soil and sieved using 2 mm sieve

was placed. The treatments consisted of four different combinations of plant growth promoting bacteria with AMF. The microbial inoculants used in the experiment are *Azotobacter* (nitrogen fixer), NPK liquid consortia (*Azotobacter, Azospirillum,* phosphate solubilizers and potassium solubilizers), Arbuscular mycorrhiza fungi, Zinc solubilizing bacteria and CRC-4 (*Advenella spp).*

The microbial strain CRC-4 (Advenella *spp)* was obtained from the NCBI GenBank (HQ995501). The bacterial culture was multiplied in Pikovskayas broth. The bacterial suspension was centrifuged at 5000 rpm for 20 minutes. The supernatant was discarded and the pellet is collected. After collection, the pellet was added to 80% NaCl solution. The NaCl + Pellet solution was added to autoclaved vermicompost which is used as a carrier for the bioinoculant. The other bioinoculant *Azotobacter* (nitrogen fixer), NPK liquid consortia (*Azotobacter, Azospirillum,* phosphate solubilizers and potassium solubilizers), Arbuscular mycorrhiza fungi and zinc solubilizing bacteria were taken and added to water separately. Planting material of equal size with healthy, erect shoots and roots of *Pelargonium graveolens* was taken and dipped into the water containing the bioinoculant for 40 minutes to allow the bioinoculant to colonize the root and then after 40 minutes the planting material were taken from the bioinoculant solution and transplanted in pots.

Crop raising: Quality planting material of *Pelargonium graveolens* L. was collected from the CSIR CIMAP gene bank in Lucknow. During cropping periods, routine agronomic practices for crop cultivation were followed. Twenty five days old, healthy and uniform seedlings of *Pelargonium graveolens* L. var. CIM-BIO-171 were procured from the nursery area of CSIR-CIMAP, Lucknow and transplanted on flat beds at 50 cm x 45 cm spacing in 2ndweek of January 2020, respectively. A light irrigation was provided immediately post transplantation. In all pots about 20 g each of AMF and bacterial cultures were applied as per treatments. All the recommended cultural practices like irrigation, weeding and hoeing were followed according to the requirement during crop growth period. Non inoculated control pots were fertilized with required amount of N, P, K through vermicompost.

Evaluation of growth and yield: At maturity, observation related to plant growth parameters were recorded per plant. Plant height was recorded from the base to the tip of the stem. Number of branches per pot was also counted. Fresh geranium biomass was observed from each treatment during harvesting, and the weight of the sampled plant was also included to the treatment.

Essential oil extraction: The oils were extracted from 200 g of each sample by hydro-distillation using Clevenger-type apparatus for 3 h. For the experiment, a 2 l flask was used (Clevenger 1928). The water volume had ratio of 1:10 (w/v), with the water condensation maintained between 12 and 15°C using the same refrigeration system (Oliveira et al 2019) for each oil. The resulting oils were centrifuged for 5 min at 3000 rpm, dried using anhydrous sodium sulfate (Na_2SO_4), then centrifuged again under the same conditions, after which the solutions for chromatographic analysis were immediately prepared. Total oil yields were expressed as essential oil (g) / dried material (g).

Quality analysis: The quality analysis of essential oil was done by gas chromatography (Helwlett Packard G.C.; H.P-5890) using FID and 15mmx 0.53 mm, B.P-20 capillary columns. Oven temperature was maintained from 40 and 220°C @ 5/ min. with initial hold of 5min. and hydrogen gas was used as a carrier at 30 ml/min. Temperature of injector and detector were maintained at 200 and 240°C, respectively. H.P.3396 integrator was used for data processing. The retention index was calculated for all volatile constituents using a homologous series of n-alkanes (C8 - C20), and were identified by comparing the experimentally obtained mass spectra and retention indices to those found in literature (Adams 2007). Major compounds viz., Linalool, isomenthon, citronellol, geranial, citronellyl formate, geranyl formate and 10-epi-Ɣ-eudesmol were identified based on the retention time of standard compound.

Soil analysis: For determination of nutrient balance of soil, the initial and post harvest phase soil samples were collected from depth of 0-15 cm separately for microbial and soil nutrient analysis from three randomly selected sited from each plot. Freshly obtained samples of soil were used for microbial and enzymatic activity as well as rest soil samples were mixed, homogenized, air dried, crushed properly, sieved (2mm) and a amalgamated sample of approximately 550 g obtained. For determination of enzymatic activity in soil viz., soil dehydrogenase activity was tested (Casida et al 1964) as well as acid and alkaline phosphatase test (Tabatabai and Bremmer 1969, Eivazi and Tabatabai 1977). Soil microbial biomass carbon (SMBC) was determined by fumigation-extraction method (Vance et al 1987). The concentration of elements in soil samples and its physical and chemical properties were measured. Organic carbon was determined (Walkley and Black 1934), available nitrogen of soil was measured by Kjeldal method (Subbiah and Asija 1956), available phosphorus by Olsen method (Olsen et al 1954), available potassium by normal ammonium acetate using flame photometer (Jackson 1973).

Statistical analysis: Statistical data analysis was analyzed by using OPSTAT statistical software package.

RESULTS AND DISCUSSION

Soil biological properties: Initial count of bacteria, fungi and actinomycetes were 27.3 x 10^4, 20 X 10^6 and 10 x 10^4, respectively before any treatment was applied in soil of experimental pot. At 30 days after inoculation (DAI), the highest bacterial population was in the rhizosphere soil of treatment T1 having AMF + CRC-4 as microbial inoculants i.e. 15.35 x 10^5 Cfu /g of soil, which was significantly higher than non inoculated control (12.8 x 10^5 Cfu /g of soil).

Soil microbial enzymes: The soil nutrient status as well as microbial enzymes were significantly influenced by co-inoculation of different plant growth promoting bacteria with arbuscular mycorrhiza fungi (AMF). T1 treatment (co inoculation of AMF and CRC-4) recorded the highest **s**oil microbial biomass carbon (SMBC) which was (162.68 µg g^{-1} soil), while all other treatments also recorded significantly higher SMBC than non inoculated control (28.43 µg g^{-1} soil). Soil microbial biomass is necessary to sustain soil functions because it serves as a source of soil enzymes that govern the transformation processes essential elements in soil and also regulates the accumulation and decomposition of organic materials, as well as the breakdown of organic residues, and acts as an early marker of soil management changes. The microbial biomass is a living component of soil that aids in nutrient transformation and cycling and also serves as a chief source of C, N, P, and sulphur, as well as enhances the physico-chemical characteristics of soil. The higher values of SMBC in soil with microbial inoculation indicate a positive trend. Dehydrogenase is a respiratory chain enzyme of soil microorganism, which is an indicator of biological redox system and microbial activity (Suryavanshi et al 2016). Dehydrogenase enzyme was significantly higher in all the treatment combinations ranging from 155.6 µg TPF g^{-1} in T4 (coinoculation of AMF and azotobacter) to 151.7 µg TPF g^{-1} soil h^{-1} in T3 (coinoculation of AMF and azotobacter) as compared to non-inoculated control (109.9 µg TPF g^{-1} soil h^{-1}). Dehydrogenase (DHA), which is primarily an intracellular enzyme, acts as a measure of metabolic activity undertaken by microbial community in soil. Acid and alkaline phosphatase also followed similar trend where all treatments recorded significantly higher values than non inoculated control.

Table 1. Effect of microbial bio-elicitors on general microbial population of soil at 30 days after inoculation in rhizospheric soil

Treatments	Population of microbes in rhizospheric soil (Cfu /g of soil)
T1(AMF +CRC4)	15.35
T2 (AMF +NPK consortia)	14.05
T3 (AMF + Zinc solubilizing bacteria)	13.65
T4 (AMF +Azotobacter)	13.80
Non inoculated control	12.80
CD (p=0.05)	0.88

Soil nutrient status: Available nutrients in soil was significantly influenced by microbial inoculants. The available N ranged from 455 kg ha^{-1} in T4 to 155 kg ha^{-1} in non-inoculated control (Table 2). Similarly all the inoculated treatments recorded significantly higher available P, K and organic carbon than control. The maximum organic carbon content was also recorded in T1 i.e. inoculation of AMF with CRC-4 (0.72 kg ha^{-1}) and the lowest were in control (0.53 kg ha^{-1}). Ravikumar et al (2012) observed that simultaneous application of biofertilizers helped in improving the availability and absorption of nutrients by the plants, resulting in the highest NPK status and uptake in plants. Enhanced activity of microbial enzymes might have lead to increased accessibility of nutrients (N, P and K). Increase in microbial biomass carbon due to dual inoculation of AMF with plant growth promoting bacteria perhaps resulted in increased organic matter content. Plant growth promoting bacteria might have stimulated better root growth leading to enhanced nutrient availability.

Crop growth parameters: The tallest plants were recorded in T1 treatment i.e., co-inoculation of AMF with CRC 4

Table 2. Effect of microbial bio-elicitors on nutrient status and soil microbial enzymes after harvesting rose scented geranium

Treatment	Available nutrients (kg ha^{-1})				Soil microbial enzymes			
	N	P	K	OC	Dehydrogenase (µg TPF g^{-1} soil h^{-1})	Acid phosphates (µg PNP g^{-1} soil h^{-1})	Alkaline phosphatase (µg PNP g^{-1} soil h^{-1})	SMBC (µg g^{-1} soil)
T1	325	17.95	200.24	0.725	153.45	98.90	170.3	162.68
T2	315	19.50	214.48	0.71	136.9	82.28	164.74	161.82
T3	378	22.18	154.84	0.65	151.7	71.13	173.8	94.39
T4	455	18.06	180.6	0.61	155.6	55.05	164.4	76.47
Control	161	10.37	125.44	0.53	109.9	32.11	85.53	28.43
CD (p=0.05)	1.1	0.03	0.3	5.7	2.7	5.7	0.6	0.4

(50.0cm), which was significantly higher than control (30.0 cm) (Table 3). Plants of T2, T3 and T4 were of almost similar height. Number of branches per plant also followed similar trend. The crop of the control plots resulted in production of plants with least height which were significantly inferior to other treatments. This might be due to fact that co-inoculation of AMF with plant growth-promoting bacteria may have increased the rate of mineralization of plant nutrients in soil, resulting in improved crop nutrition and consequently increased plant height. Similar results were reported on potato by Talwar et al (2016) in onion and Thakur et al (2016) in calendula.

Dry matter yield: Inoculation with microbial bio-elicitors significantly enhanced the dry matter production of rose scented geranium over non inoculated control (Table 4). T1 (AMF+CRC 4) recorded the highest total dry matter yield (23.79 g/plant) closely followed by T2 (18.52 g/plant), while the lowest dry matter yield was recorded in non inoculated control (9.22 g/plant). Improvement in herb yield was accredited to sustained availability of macro and micronutrients for longer period of crop growth which is related to enhanced activity of microbial enzymes. The co-inoculation of AMF with plant growth promoting bacteria recorded relatively larger percentage of dry matter integrating into leaves in comparison with untreated control at harvest stage. The results indicated the need of microbial inoculation for improving dry matter partitioning into the geranium leaves. This could be because colonization with AMF increased nutrient and water intake, resulting in increased absorbing area via mycelium infiltration of the soil and an increased soil volume for the plant, resulting in higher photosynthesis, plant development, and plant weight gain (Smith et al 2003).

Essential oil yield: Oil content of microbial inoculated plants ranged from 0.35% in T1 to 0.28% in T4, which were significantly higher than non inoculated control (0.19%). Co-inoculation of CRC-4 with AMF was very effective in increasing oil yield of geranium. This might have happened due to increased availability of nitrogen and phosphorus in rhizosphere of inoculated plants. Higher photosynthetic activity might stimulate enhancement in production of essential oil yield, which is a secondary metabolite. Copetta et al (2006) also reported increase in essential oil yield due to changes in phytohormones leading to higher number of pellate grandular trichomes in the leaves as a result of inoculation. Similarly, Bajeli et al (2016) in Japanese mint and Singh et al (2014) in basil reported an increase in essential oil yield due to application of organic manure.

Oil composition: GC-MS results reported the major components of geranium oil as citronellol (23.15-32.56%), geranial (8.48-12.85%), citronellyl formate (5.94-9.89%),

Table 3. Effect of microbial bio-elicitors on aerial and root dry matter and essential oil content of rose scented geranium

Treatment	Height (cm)	No. of branches /plant	Dry matter (g/plant)			Total dry biomass (g/plant)	Essential oil percent (%) per 200 g plant biomass
			Aerial shoot		Below ground		
			Leaf	Stem	Root		
T1	50.0	13	18.85	4	0.94	23.79	0.35
T2	41.25	10.5	14.75	2.9	0.85	18.50	0.31
T3	40.25	10.5	11.3	2.8	0.58	14.68	0.30
T4	39.75	9.75	15	1.25	0.50	16.75	0.28
Control	32.0	9.0	7.9	1.0	0.32	9.22	0.19
CD (p=0.05)	0.23	0.12	1.0	1.04	1.2	0.003	0.09

Table 4. Effect of microbial bio-elicitors on chemical composition of essential oil of rose scented geranium

Compound	T_1	T_2	T_3	T_4	Control
Linalool	1.02	0.87	1.80	1.00	2.06
Isomenthon	6.16	7.92	7.96	7.28	6.65
Citronellol	32.56	23.15	32.62	31.24	26.56
Geranial	12.85	8.48	10.67	9.43	8.43
Citronellyl formate	9.32	5.94	9.77	9.89	7.51
Geranyl formate	2.83	1.68	2.39	2.81	1.11
10-epi-Y-Eudesmol	6.98	5.99	6.08	6.90	3.72

isomenthon (6.1-7.96%), 10-epi-Y-Eudesmol (-6.98%) and linalool (0.87-2.06%). Co inoculation of plant growth promoting bacteria along with AMF gave encouraging results. T1, T3 and T4 recorded almost similar amount of citronellol, citronellyl formate, geranyl formate and 10-epi-Y-Eudesmol, which was significantly higher than untreated control. As citronellol content defines the commercial value of geranium oil, co-inoculation of AMF with CRC-4 (T1), Zinc solubilizing bacteria (T3) and Azotobacter (T4) had synergistic effect which resulted in improvement in desirable constituent in oil. Root colonization with AMF enhanced the EO content and composition as reported by Morelli et al (2017) in basil and Trindade et al (2019) in *Piper nigrum*.

CONCLUSION

Natural bio-elicitors, such as dual inoculation of arbuscular mycorrhizal fungi with plant growth promoting bacteria, can help preserving the ecological balance of the soil while enhancing yield and quality of essential oil of rose scented geranium. As a result, the use of microbial inoculants in conjunction with inorganic chemical fertilizers can minimize reliance on inorganic chemical fertilizers, potentially paving the way for sustainable and organic production of aromatic and medicinal plants.

ACKNOWLEDGEMENT

Authors acknowledge the funding support from CSIR AROMA Mission (HCP 007) for providing necessary funding.

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Received 22 March, 2022; Accepted 20 May, 2022

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