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Effect of Indole Acetic Acid Producing Bacteria and Hormone Applications on Essential Oil Components of *Satureja hortensis* L

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Abstract: The impacts of plant growth-promoting bacteria on aromatic plant species are poorly known. This study was conducted in order to evaluate possible impacts of mineral fertilizer, plant growth regulators [PGR, Indole 3-acetic acid + benzylaminopurine IAA+BAP and BAP] and five N_2-fixing, P-solubilizing, IAA-producing bacterial strains, as biofertilizer on growth, yield and quality characteristics of the plant, essential oil content and major component of the essential of summer savory. GC-MS was used to analyze the hydro-distilled essential oils. Multi-traits bacterial inoculation and PGR applications stimulated overall plant growth, including drug and fresh herb yield, essential oil ratio and yield, crucial oil components such as carvacrol of savory. The presence of at least thirty one compounds were identified in analysed samples, representing 98.7-100% of the total oil contents. In terms of general categories, the chemical profile of the oils with carvacrol, p-cymene, γ-terpinene and α-terpinene as the basic components was dominated by monoterpene hydrocarbons.

Keywords: Plant Growth-Promoting Rhizobacteria, Plant Growth Regulators, Summer Savory, Essential Oil Component and Yield

1. Introduction

The plant hormones are important for the production of essential oil-bearing crops. The concentration, application time and type of plant growth regulators [PGR] are a crucial factor for the metabolism and accumulation of secondary metabolites and can affect the essential oil content [1, 2]. Summer savory (*Satureja hortensis* L.) can be used as a good alternative to conventional synthetic antimicrobial additives in foods [3] and it is widely used in the food, herbal teas, spices, beverage, flavoring material, medicine, drink and perfume industries in the world [4-6]. The essential components of savory are generally phenolic compounds such as carvacrol and thymol, including gamma-terpinene, β-caryophyllene, p-cymene, linalool and other terpenoids [4, 7]. Some investigators have determined that the oil components of the plant are thymol, carvacrol, γ-terpinene, and borneol [8]. In addition to its application as a natural food preservative and spice, *S. hortensis,* an annual aromatic herb, is known to have been used in the conventional folk medicine in the applications of cardiovascular diseases [9] and muscle pain reliever, intestinal and stomach disorders [10]. Essential oils (EO) and extract of this plant species have antioxidant [11-13], antibacterial-antimicrobial [7, 11, 13-15], insecticidal [16, 17], antifungal [11, 18-20] and antigenotoxic activities [21].

Plant growth-promoting rhizobacteria [PGPR] inoculation may be an effective biotechnological tool that stimulates the mechanism of the secondary metabolite production in plants [22]. On the other hand, using different PGR to improve EO yield, composition and content, research that will create an opportunity to change and/or improve the composition of some molecules having economic interest gains importance [23]. Several important bacterial characteristics, such as

solubilization of inorganic phosphate, biological nitrogen fixation, mineralization of organic matter and solubilization of inorganic phosphate, secretion of certain volatile organic compounds and lowering of plant ethylene level, nutrient uptake, promoting beneficial plant-microbial association, increasing root permeability, production of phytohormones and siderophores, 1-aminocydopropane-1-carboxylic acid deaminase activity, and exhibition of antimicrobial activity against phytopathogenic microorganisms by several mechanisms, can be evaluated as plant growth promotion features and promoted directly or indirectly by many mechanisms such as plant development [23-28]. By efficient use, it is expected that PGPR will make contribution to agronomic efficiency, commonly by reducing environmental pollution and costs, by removing the harmful chemicals.

Agricultural practices can change oil content and composition more or less in medicinal and aromatic plants, PGPR applications are essential for oil synthesis and its role in secondary metabolite production processes are less well known and bacterial IAA and plant growth regulators applications need to be investigated in different medicinal and aromatic plant species. The use of bacterial IAA in medicinal and aromatic plants is an important area of research for increasing the amount of EO and its components. In particular, the possibilities of medicinal and aromatic plants yield, EO content and components to be increased or replaced must be extensively investigated using IAA-producing bacteria. Although some experiments have addressed the role of PGPR inoculation in aromatic plants [25, 29, 30], the impact of PGPR on physiological features and production of secondary metabolites is poorly known [21]. Therefore, for improve growth and development of these plants whit on native PGPR strains as inoculants have increased significantly day by day research. While our knowledge regarding the potential of PGPR to affect the secondary metabolic pathways of aromatic and medicinal plants remains fragmentary, there are no studies about the effects of theirs on summer savory growth, yield and essential oils. In addition, there is a trend towards biological fertilizer applications and sustainable agricultural systems in the production of medical plants due to environmental impacts which are caused by over application of chemical fertilizers, energies and expenses. That is why, a study was carried out in order to investigate the impact of plant growth regulators and different N_2-fixing, IAA-producing, and P-solubilizing bacterial species on growth and development, yield, content and composition of essential oils of summer savory.

2. Materials and Methods

2.1. Bacterial Strains

Five varied potential PGPR from a pool of 320 rhizobacterial isolates obtained from the oregano (*Origanum rotundifolium*) and wild raspberry [*Rubus ideaus*] rhizosphere on the basis of their P-solubilizing, N_2-fixing and IAA-producing ability were chosen. Sources and some of biochemical characteristics of the bacterial strains used in the study were given in Table 1.

Table 1. *Isolates used in experiments and some properties.*

Bacterial strains†	Source	Gram reaction	Oxidase	Catalase	Sucros	IAA-production	N_2-fixation	P-solubilization
P. fluorescens RC26	Raspberry	-	+	+	+	S+	+	+
M. luteus RC17A	Raspberry	+	+	+	-	S+	S+	+
B. subtilis RC17C	Raspberry	+	W+	+	+	S+	S+	+
B. subtilis RC631	Oregano	+	+	+	+	+	S+	W+
P. putida RC48	Oregano	-	+	+	+	+	+	+

†Pseudomonas fluorescens RC26, Micrococcus luteus RC17A, Bacillus subtilis RC17C, Bacillus subtilis RC631, Pseudomonas putida RC48, +: positive; S +: strong positive, W +: weak positive

2.2. Greenhouse Experiment, Inoculation and Growth Conditions

The experiments were carried out a completely randomized design with six replicates (each having four seedlings), having 9 treatments as (1) Control (without bacteria or mineral fertilizers), (2) NPK fertilizer (300 mg N + 300 mg P + 300 mg K plant in the compound fertilizer), (3), benzylaminopurine (BAP, 100 ppm), (4) indole acetic acid + benzylaminopurine (IAA+BAP, 100 ppm), (5) *Pseudomonas fluorescens* RC26, (6) *Pseudomonas putida* RC48, (7) *Micrococcus luteus* RC17A, (8) *Bacillus subtilis* RC17C and (9) *Bacillus subtilis* RC63. The experiment was repeated twice. 20% sodium hypochlorite solution and filled with a loamy soil with an organic matter content of 3.1% (w/w), a pH of 7.4, total N of 0.16%, an available Olsen-P content of 17.4 mg kg^{-1}, and available K, Ca and Mg contents of 426, 3260 and 468 mg kg^{-1}, respectively were used to sterilise the pots. Available Fe, Zn, Mn and Cu contents were 5.9, 9.3, 1.3, and 1.6 ppm, respectively. Chemical fertilizer application is homogeneously mixed during the filling of the sieved soil with the pot.

At the beginning of study, the seed of *S. hortensis* were sown in greenhouse on March, seed pillows of 1 x 1.5 m containing sand, peat and virgin soil (1: 1: 1). After two month from sowing, the seedlings with 5-8 cm height were transplanted to the pots. Pure cultures (50% strength TSB) were growned in rotary shaker (120 rpm; 25 °C) for three days. Bacteria which harvested with centrifugation (ca. 3000 x g for 10 min) washed and re-suspended in 10 mM sterile phosphate buffer [SPB]. Media pH is set to 7 for bacteria. . Every one of the PGPR strains is used to vaccinate uniform height young 2-month-old seedlings. Prior to planting for 60

min, root system of seedlings was plunged into a suspension of every one of PGPR strains for the bacterial inoculation. [31]. Control plants were placed in 5 ml of diluted SPB with no bacteria. Sixty-day-old seedlings of savory were transplanted to the pots. The savory seedlings were grown in a greenhouse under a day-night cycle of 13–11 h natural light (18°C-14°C) and 60% relative humidity. Initially, after free drainage had stopped, the pots were measured in order to calculate the mean mass for each soil at field capacity. Pots were watered until reaching up to 100% of their field capacity and the amount of water diminished every 4 days was applied to the pot. The herbs were manually taken when the weeding is necessary.

2.3. Plant Material and Isolation of Essential Oil

The plants were harvested at a height of 5-6 cm from the ground surface at 50-60% of the flowering phase and dried in shade. Plant samples which are dried were exposed to plant material which is in boiling water hydro distillation for 3 hours using a Clevenger-type apparatus. Distillation was repeated 3 times for each sample.

2.4. GC–MS Analysis

The analysis of the essential oil was performed with gas chromatography-mass spectrometry using the Ultra Thermo desorption unit connected to a quadrupole GC-MS-QP2010 Ultra (EI Quadrapole, Shimadzu, Kyoto, Japan equipped with a HP-5 standart MS fused silica capillary column). This analysis was made according to Adams, R. P. [32].

2.5. Statistical Analysis

The experiment was repeated twice. The data were determined according to analysis of variance using SPSS 10.0 software package and when the medium was determined, Duncan's Multiple Range Test was used.

3. Result

All vaccinated strains of PGPR were able to produce plant growth–promoting phytohormones, solubilize P, and showed nitrogenase activity (Table 1). Dry herb weight in plants vaccinated with three of the tested bacterial strains was considerably higher than in controls (Table 2). Of the bacterial inoculants, maximum dry herb weight in savory was measured in the N_2-fixing, IAA-producing and P-solubilizing *Micrococcus luteus* RC17A and NPK fertilizer, followed by *Pseudomonas fluorescens* RC26, *Bacillus subtilis* RC17C, and exogenous application of indole acetic acid + benzyl amino purine (IAA+BAP) combination, while statistical difference was not seen between the other treatments in terms of dry herb weight.

When compared with the control, average NPK, BAP and IAA+BAP applications increased the essential oil (EO) content, respectively, by 14.6, 10.7 and 24.1%, while inoculations with PGPR by 11.3-32.8%. In this study, the EO content of summer savory varied from 2.12-2.82% according to the treatments, while in previous studies on cultivated savory EO was found 1.75-2.3% [33], 1.30-2.67% [34], 0,87-1,06% [4], 2.05% [35], 0.5-2.9% [36], 1,66–2,20% [37], 2,78% [38] and 1.8-2.0% [39].

Except for cytokinin [BAP] and *P. putida* RC48, all treatments increased EO content, and EO and carvacrol yield in dry herb material of *Satureja hortensis* plants significantly compared to the control; the maximum EO and carvacrol yield in savory were found in RC26 inoculation, followed by RC17A, IAA+BAP, and RC17C. Also, addition of IAA with the BAP increased these characters more than BAP treatment alone. On an average of both trials, seedling inoculation of summer savory with RC26, RC17A, RC17C, RC631, and RC48 increased dry herb yield per plant by 17.4, 23.5, 16.4, 4.2, and -3.0% as compared to the control and EO content by 29.2, 18.9, 20.8, 33.0, and 11.3%, respectively. NPK and IAA+BAP applications, however, increased dry herb yield up to 18.5 and 13.5% and essential oil content by 14.6 and 24.1%, respectively. The data suggest that inoculation of summer savory with PGPR increased EO yield per plant by 8.0-51.4% as compared to the control, and increased carvacrol yield by 3.2-57.2%, depending on the strains. NPK, BAP, and IAA+BAP applications, however, increased EO yield up to 36.7, 8.0, and 40.3, and carvacrol yield by 32.6, 13.4, and 46.5%, respectively.

According to GC-MS analysis results, *S. hortensis* was found to contain 98.7-100% of the total of 31 components depending on the applications of volatile oil. The main components of hydrodistilled oil from aerial parts of *S. hortensis* were carvacrol (48.18-53.96%), γ-terpinene (23.76-31.11%), p-cymene (4.25-7.85%) and α-terpinene (3.91-6.24%) followed by β-myrcene (1.56-2.73%), β-bisabolene (0.67-3.13%), α-thujene (0.79-2.13%), α-pinene (0.58-2.13%), β-pinene (0.41-2.25%), β-caryophyllene (0.41-1.87%), α-phellandrene (0.38-0.70%) and spathulenol (0.26-0.67%) with lower percentages, the amount of all remaining oil constituents was less than 1%, or not found in all samples. According to many studies, carvacrol and γ-terpinene were considered as the main constituent in oils of *S. hortensis* [4, 33, 35-37, 38, 40-42].

The highest amount of monoterpene hydrocarbons (MH) in EO was obtained with the inoculation of *B. subtilis* RC631 followed by NPK and *P. putida* RC48, while the highest Oxygenated monoterpenes (MO) ratio was found to be S. hortensis inoculated with *P. fluorescens* RC26. Control and BAP treatment gave the highest sesquiterpene hydrocarbons (SH) while NPK application gave the lowest SH, bacterial vaccines having values lower than control but higher than NPK were used to apply to the plots (Table 2). The monoterpene prevalence in oil (92.64-98.01%) was evident, while the most abundant were MO (48.35-54.05%). In addition, SH (1.62 to 6.21) and SO (0.37 to 0.86%) were isolated.

Table 2. *Effect of fertilizer, plant growth regulators and rhizobacteria on essential oil content, dry herb, essential oil and carvacrol yield in summer savory.*

Applications†	Dry herb weight [g/plant] ‡	Essential oil content [%, g/100 g dry weight]	Essential oil yield [g/plant]	Carvacol yield [g/plant]
Control	17.02±0.96 c	2.12±0.10 e	0.362±0.032 b	0.187 ± 0.002 e
NPK	20.34±1.61 a	2.43±0.09 cd	0.495±0.050 a	0.248± 0.012 bc
IAA+BAP	19.32±0.93 ab	2.63±0.19 a-c	0.508±0.059 a	0.274 ±0.029 a-c
BAP	16.65±0.80 c	2.35±0.07 de	0.391±0.021 b	0.212± 0.002 de
RC26	19.98±1.05 a	2.74±0.10 ab	0.548±0.033 a	0.294± 0.010 a
RC17A	21.02± 1.35 a	2.52±0.21 b-d	0.527±0.024 a	0.281± 0.013 ab
RC17C	19.81±1.85 a	2.56±0.19 b-d	0.507±0.044 a	0.264± 0.016 a-c
RC631	17.73±0.81 bc	2.82±0.18 a	0.500±0.040 a	0.241± 0.027 cd
RC48	16.51±1.65 c	2.36±0.14 de	0.391±0.058 b	0.193± 0.030 e
Average	18.60±2.00	2.50±0.25	0.470±0.076	0.243± 0.403

†Control: without bacteria inoculation or mineral fertilizers; Bacterial strains are explained in Table 1;
‡Values followed by different letters in a column were significantly different [$p < 0.05$], using Duncan's multiple range test; Data were means ± standard error of six replicates.

The biosynthesis of the major EO constituents was enhanced in the vaccinated plants. The maximum carvacrol and spathulenol in EO were obtained with treatment of IAA+BAP, BAP and inoculation RC26, and γ-terpinene and p-cymene in EO were obtained with NPK fertilization and RC48 inoculation, respectively. IAA + BAP resulted in an increase in the yield of EO and carvacrol without much influence on the main compounds, but with some change in the composition such as the increase and appearance of [α-thujene, β-myrcene, carvacrol, limonene, and spathulenol] and reduce of [p-cymene, β-caryophyllene and β-bisabolene]. All five PGPR strains evaluated produced a significant increase in α-thujene, β-myrcene and α-terpinene content, but only RC17A, RC17C and RC631 strains and NPK treatment enhanced γ-terpinene, and only RC48 increased p-cymene content. In this study, α-thujene, α- pinene, α-terpinene, β-pinene, p-cymene and γ-terpinene were the most influenced monoterpenes. While the highest β-caryophyllene and β-bisabolene rate in oil was determined in control application, other components were changed with bacteria inoculations. Moreover, the use of plant growth regulators (IAA+BAP) and inoculation with RC17C and RC48 caused the appearance of limonene and disappearance of sylvestrene.

Table 3. *Effect of mineral fertilizer, rhizobacteria and plant growth regulators on essential oil components of summer savory.*

Compounds	Treatments‡								
	Control	NPK	IAA+BAP	BAP	RC26	RC17A	RC17C	RC631	RC48
α-thujene	0.87 d	1.46 b	1.10 c	0.79 d	1.25 c	1.14 c	1.25 c	2.09 a	2.13 a
α- pinene	0.65 de	0.76 d	0.58 e	0.64 de	1.06 c	0.70 de	0.78 d	1.31 b	2.13 a
Camphene									0.20
Sabinene		0.12			0.15				
β-pinene	0.47 d	0.46 d	0.47 d	0.53 cd	0.46 d	0.41 d	0.60 c	0.91 b	2.25 a
β-myrcene	1.64 c	2.21 b	2.03 b	1.56 c	2.73 a	2.04 b	2.05 b	2.73 a	2.26 b
α-phellandrene	0.46 b	0.53 b	0.49 b	0.38 c	0.64 a	0.48 b	0.50 b	0.70 a	0.51 b
δ-3-carene		0.10							0.12
α-terpinene	4.25 e	5.23 bc	4.75 cd	3.91 e	5.31 b	4.57 d	4.79 b-d	6.24 a	4.52 d
p-cymene	5.56 b	4.99 c	4.81 c	4.30 d	4.37 d	4.25 d	5.12 c	5.64 b	7.85 a
Sylvestrene	0.34	0.82		0.60		0.79		1.00	
Limonene			0.77				0.79		1.02
β-phellandrene					0.45				
[Z]- β-ocimene									0.37
γ-terpinene	25.59 cd	31.11 a	27.54 bc	25.97 cd	26.06 cd	28.67 b	28.59 b	28.61 b	23.76 d
Trans-sabinene hydrate	0.04	0.11		0.18			0.20	0.17	
α-terpinolene									0.20
Borneol	0.17								0.17
α-terpineol									0.14
Carvacrol methyl ether	0.65	0.09			0.16	0.12		0.17	0.65
Carvacrol	51.89 ab	50.02 bc	53.92 a	53.96 a	53.89 a	53.05 ab	51.91 ab	48.18 c	49.72 bc
Carvacrol acetate	0.05					0.22	0.28		
β-caryophyllene	1.87 a	0.59 d	0.95 bc	1.80 a	0.96 bc	1.10 b	0.97 b	0.80 c	0.41 e
Aromadendrene	0.35		0.30	0.53					
γ-selinene									0.07
Bicyclogermacrene	0.77			0.49	0.53			0.22	0.48
β-bisabolene	3.13 a	0.73 e	1.62 c	1.94 b	1.43 cd	1.41 cd	1.20 d	0.83 e	0.67 e
Cis- α-bisabolene	0.09			0.21		0.14			
Spathulenol	0.52 b	0.26 d	0.67 a	0.57 b	0.53 b	0.50 b	0.52 b	0.40 c	0.37 c
Caryophyllene oxide	0.10	0.10		0.30		0.20	0.15		
3-fluorophenyl	0.04	0.22				0.16	0.14		
MH†	39.87 d	47.90 ab	42.54 cd	38.86 d	42.48 cd	43.05 cd	44.67 bc	49.40 a	47.32 ab

Compounds	Treatments‡								
	Control	NPK	IAA+BAP	BAP	RC26	RC17A	RC17C	RC631	RC48
MO	52.77 ab	50.11 ab	53.92 a	53.96 a	54.05 a	53.39 a	52.19 ab	48.35 b	50.68 ab
SH	6.21 a	1.32 f	2.87 c	4.97 b	2.92 c	2.65 c	2.17 d	1.85 de	1.63 ef
SO	0.67 b	0.58 c	0.67 b	0.87 a	0.53 c	0.86 a	0.81 a	0.40 d	0.37 d
Total identified	99.52	99.91	100	98.66	99.98	99.95	99.84	100	100

†MH: Monoterpene hydrocarbons; MO: Oxygenated monoterpenes, SH: Sesquiterpene hydrocarbons; SO: Oxygenated sesquiterpenes, ‡Different letters within the same line indicate significant differences according to Duncan's Multiple Range Test [$p \leq 0.05$].

4. Discussion

It is clear that the influence of PGR and PGPR on the both EO content and its composition are the most important quality criteria for summer savory in all purposes. The production of auxin (IAA), has been accepted as a significant element in direct plant-growth-promoting abilities of rhizosphere bacteria [43]. Also exogenous application of PGR could affect EO production and chemical compositions. Plant growth regulators have been determined as one of the main elements influence plants growth and their primary and secondary metabolites pool, and could affect EO production and chemical composition. The BAP and IAA caused an increase in the yield of EO of *O. gratissimum* without much influence on the main compounds [23], but with some change in the composition. Application of auxin and cytokinin increased some components, medicinal and nutritional values of the lemon balm and verbena [44, 45], enhanced callus induction and shoot and root formation in *in vitro* culture of lemon balm [46] and volatile compounds such as thymol of thyme [47]. On the other hand, in the previous survey , it can be seen that the spraying with BAP on plants leads to an increase of the plant weight, leaf density and area of the glandular hairs [22].

In this study we are trying to examine the effects of different PGPR on yield, content and composition of EO in order to have opportunity to develop and/or change the composition of some molecules having economical interest. Moreover, qualitative and quantitative changes in the major monoterpene components and sesquiterpene content of plant oil were observed in response to the effect of varying growth regulators and bacterial strains, by the host plant in a strain-specific attitude. Exogenous application of cytokinin (BAP) and auxin (IAA) have the effect of increasing the yield of EO without considerable qualitative change.

Bacterial inoculation affected the chemical composition of EO and increased the production of monoterpenes. The inoculated plants, in comparison with controls, showed increases yield, composition and content of EO, and marked qualitative and quantitative changes in some molecules having economical interest. Recent studies have shown that bacterial inoculation affected density, size, number, formation and interfered with the chemistry of glandular trichomes which probably enhanced the EO synthesis [48, 49]. PGPR inoculations, a potential alternative in plant production, had an increased oil content and yield in calendula [50], induced biosynthesis of secondary metabolites, and affected pathway flux or specific steps of monoterpene metabolism [25], and improved the production of monoterpenes and main constituents [21, 25, 29, 45, 51, 52]. It has been found that PGPR significantly enhances in root and shoot biomass, node number, leaf area, glandular trichome number and density, and stomatal density, EO yield and biosynthesis of major EO components, and marked qualitative and quantitative changes in monoterpene content of peppermint [21, 52], produce high density and large of glandular trichomes, it could be suitable for secondary metabolite production of yellow lapacho [53], increase total EO yield and phenolic content in oregano and thyme [30, 51], and increase root and shoot dry weight, P, N and K content, and EO of sweet basil [54]. The positive impacts of these strains on growth, yield, oil content and compositions of summer savory plants indicates the useful role of these PGPR, which might be attributed to IAA production, P-solubilisation, N_2-fixation or even other non-evaluated PGPR traits that stimulate plant.

5. Conclusion

Improved knowledge of the factors that control or affect monoterpene accumulation and biosynthesis of secondary metabolites will cause strategies for advanced productivity and cultivation of aromatic plants without the use of chemical fertilizers or PGR. PGPR have clear potential for improving the productivity of aromatic plants, and may significantly enhance plant growth and diminish the amount of fertilizers needed for economically sustainable crop production. Bacterial inoculants may be an efficient biotechnological tool for stimulating secondary metabolism in medicinal and aromatic plants, and studies of their activities will enhance our understanding of processes that affect the accumulation of phenolic compounds and monoterpenes for a variety of treatments in food and cosmetic industries, and poorly understand at present. Microbial strategy presents an attractive way to replace the use of chemical fertilizers for aromatic and medicinal plants, but little is known about their potential effect and ability of PGPR to increase plant secondary metabolites. Multi-traits PGPR inoculation can significantly modify both the content and composition of EO in savory, and seem to be an important factor modifying their aromatic profile. Also, more studies are necessary to examine the probable mechanisms by which bacteria enhance phytochemical constituents in medicinal plants at the tissue, cell, or molecular level.

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