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Influence of Drought Stress and PGPR Inoculation on Essential Oil Yield and Volatile Organic Compound Emissions in *Mentha piperita*

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Abstract: Considering that inoculation with beneficial rhizobacteria promotes biomass and secondary metabolite biosynthesis and also the fact that drought stress can increase their production, we determined the effects when both of these situations were combined in EO yield. In addition, the levels of endogenous phytohormones and the gene expression of enzymes related to monoterpenes biosynthesis were measured. The experimental results showed that inoculation with PGPR in stressed plants produced the same effects on trichome density, the EO main components and total yield as in plants only inoculated or with moderate stress alone. In addition, the VOC emissions of inoculated stressed plants did not vary the release amount in relation to uninoculated stressed ones. The results observed have suggested a way of improving aromatic plant productivity, particularly that of peppermint, by managing drought stress through the inoculation of plants with PGPR. This inoculation under moderate drought stress is appropriate when the purpose of the crops is to obtain plants with a high secondary metabolites yield.

Keywords: drought stress; essential oil; VOCs; PGPR; secondary metabolites

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1. Introduction

The lack of an appropriate level of water is one of the most disturbing environmental features affecting crop yield. It influences plant health by modifying multiple factors related to the metabolism of the plant, generating osmotic and oxidative stress, and ultimately causing the death of the plant due to the increase of high levels of reactive oxygen species (ROS). In addition, plant specialized metabolites can be altered by biotic or abiotic stress. The effect of deficiency of water, in aromatic and medicinal plants, may cause in some cases positive effects on secondary metabolite yield and composition [1]. Although the quantity and components of the EOs differ among regions, as a result of differences in climate, soil composition, agronomic conditions, harvesting season, essential oil processing technique and the drying system applied [2], water deficit is found to be the main stress disturbing numerous processes in the plant chemistry [3].

The essential oil (EO) production of aromatic plants is significantly connected with the glandular trichomes, which are specialized foliar organs where oil constituents are produced and stored [4]. Furthermore, phytohormones have been found to positively affect the amounts and flavor of EO, especially Jasmonic acid (JA), which control the biosynthesis of secondary metabolites, particularly terpenoids, and regulates secondary metabolite biosynthetic pathways [5,6].

Mentha piperita is an aromatic plant from the Lamiaceae family and is of economic interest due to the production essential oils (EOs) and phenolic compounds [2]. It has long been consumed in traditional medicine for the treatment of several diseases, including gastrointestinal infectious and respiratory disease, such as anti-congestive and expectorants and as anti-spasmodic on the digestive and vascular systems [7–9]. Furthermore, it is

currently one of the most important crops from the economic and pharmaceutical points of view. Its EOs have been shown to be insecticidal and demonstrate antimicrobial efficacy against bacterial and fungal plant pathogens [8].

Plant growth promoting rhizobacteria (PGPR) are soil bacteria that in addition to favoring plants growth in some cases, are able to decrease the occurrence or intensity of plant diseases by the induction of systemic resistance (ISR), a resistance mechanism that generates structural or functional modifications related with plant defense [10–12]. Moreover, in the last few years, it has been described that certain PGPR enhance the tolerance to water deficiency stress [13,14]. Our previous studies have shown that the inoculation of peppermint plants subjected to drought stress has the ability to improve various parameters of interest, such as the fresh biomass. In addition, drought effects were ameliorated by the reduction of MDA (malonyldialdehyde) levels, thus avoiding the accumulation of ROS and increasing antioxidant enzyme activities and the antioxidant level, specifically in relation to the total phenolic compounds [14].

Considering that the inoculation with PGPR promotes biomass and secondary metabolite biosynthesis, and taking into account that drought stress could also increase their production, we determined the effects of the application of both these conditions on EO yield. Thus, the present survey was designed to analyze the effect of PGPR inoculation on the biosynthesis and accumulation of EOs and in particular their emission in *M. piperita*. Additionally, we determined the amount of endogenous plant hormones and gene expression of enzymes implicated in the protein synthesis leading to bioactive terpenes, in plants subjected to drought stress and PGPR, to try to get a deeper understanding of how the influence of drought stress and PGPR inoculation in combination modifies secondary metabolite biosynthesis in *M. piperita*.

2. Materials and Methods

2.1. Plant, Bacterial Inoculation, and Treatments

The PGPR strains used were *Pseudomonas simiae* WCS417r and *Bacillus amyloliquefaciens* GB03, grown on LB medium [15], and conserved in nutrient broth with 15% glycerol at −80 °C. Strains used in the experiments belong to the Research Institute of Environmental Biotechnology and Health (INBIAS) collection (Río Cuarto city, Argentina) and were previously reported for plant growth-promoting activity [16,17]. For assays, bacteria were grown on nutrient agar, and single colonies were transferred to flasks with their culture media and then grown aerobically on a rotary shaker (150 rpm) for 24 h at 25 °C. The bacterial suspension was re-suspended in 0.9% NaCl (*w*/*v*) to a final concentration of 10^9 CFU/mL, and 1 mL of the resultant suspension was used as inoculums. Micropropagated *Mentha piperita* were obtained from a commercial crop (San José, CA, USA) grown in the San Javier department, Córdoba province, Argentina (30°30′00.0″ S, 64°30′00.0″ W) as reported by Santoro et al. [16]; the specimen was identified and deposited in the herbarium of Museo Botánico de Córdoba, Argentina (CORD 00088589). After 7 days, the plants were inoculated, and the control plants were inoculated with physiological solution. Drought stress was obtained by the suppression of water. The next treatments were performed: (a) control: normal irrigation (100% field capacity); (b) moderate stress (MS): irrigation until 10 days before harvest, (50% field capacity); (c) severe stress (SS): irrigation until 20 days before harvest, (35% field capacity); (d) WCS417r; (e) MS+WCS417r; (f) SS+WCS417r; (g) GB03; (h) MS+GB03; (9) SS+GB03.

Plants were settled randomly in a growth chamber, where conditions of light (16/8 h light/dark cycle), temperature (22 ± 2 °C) and relative humidity (∼70%) were controlled. Plants were irrigated with Hoagland's medium (30 mL/pot) two times per week [18] until starting the drought stress, at this point the SS plants did not receive more water. MS plants were irrigated with distilled sterile water until 10 d before harvest, and control plants also were watered with distilled water until being harvested.

Plant VOC (Volatile Organic Compounds) emissions were sampled 27 days after inoculation. After that, the plants were harvested, and the weight was registered. The

samples used for plant hormone quantification were lyophilized, while samples used for EO yield analysis were stored at −80 °C. Experiments were conducted three times (10 pots per treatment).

2.2. EO Extraction

The aerial part of the plant was hydrodistillated in a Clevenger-like apparatus. β-pinene was added as an internal standard (1 µL in 50 µL dichloromethane). The main EO, compounds were limonene, (-) menthone, (-) menthol, menthofurane and (+) pulegone and represent ~60% of the total EO. Chemical analyses were performed using a Perkin-Elmer Q-700 gas chromatograph (GC) equipped with a CBP-1 capillary column (30 m × 0.25 mm, film thickness 0.25 µm) and a mass selective detector. Analytical conditions: injector temperature 250 °C, detector temperature 270 °C; oven temperature programmed from 60 °C (3 min) to 240 °C at 4°/min; carrier gas = helium at a constant flow rate of 0.9 mL/min; source 70 eV. Oil components were identified based on mass spectral and retention time data in comparison to standard compounds [18]. GC analysis was performed using a Thermo Scientific TRACE 1300 gas chromatograph fitted with a 30 m × 0.25 mm fused silica capillary column coated with Supelcowax 10 (film thickness 0.25 µm). GC operating conditions: injector and detector temperatures 250 °C; oven temperature programmed from 60 °C (3 min) to 240 °C at 4°/min; detector = FID; carrier gas = nitrogen at a constant flow rate of 0.9 mL/min.

2.3. Plant VOC (Volatile Organic Compounds) Collection

The VOC collection system includes a vacuum pump that produces a continuous airflow (300 mL/min) through a polyethylene terephthalate chamber (volume 1500 mL) where a plant was placed (inoculated or uninoculated). Headspace VOCs were collected for 2 h with 30 mg Super-Q absorbent (80–100 mesh; Alltech) and analyzed according to Cappellari et al., [18].

2.4. Trichome Density

A layer of acrylic was coated onto both sides of the leaf, then meticulously removed and mounted for microscopy on a solution of glycerol/distilled water 1:10 [19]. Six leaf blades were processed for each treatment. The peltate and capitate trichomes density (number/mm^2) was determined from three microscope fields chosen at random for each leaf epidermis. Histological preparations were evaluated with a standard Zeiss model microscope. Photographs were taken with a Zeiss Axiophot microscope equipped with image capture and digitization (AxioVision 4.3, with camera AxioCam HRc 200× magnification). Peltates and capitates trichomes were counted on both sides of the leaves, using records from five microscopic fields chosen at random and observed at a magnification of 10×. Image analysis was performed using Microsoft Paint for Windows.

2.5. Quantification of Endogenous Plant Hormones

Endogenous plant hormones were extracted and quantified as described by [20]). Briefly, 100 mg of lyophilized plant material was homogenized in 1 mL methanol, and 40 ng of [$^2H_2$]JA, 40 ng [$^2H_4$]SA (salicylic acid) and 40 ng [$^2H_6$]ABA (abscisic acid) were added as internal standards. Sample analysis was performed on an Agilent 1200 HPLC system (Agilent, Santa Clara, CA, USA) coupled to an API 5000 tandem mass spectrometer (AB Sciex, Nieuwerkerk aan den IJssel, The Netherlands). Separation was achieved on a XDB C18 column (1.8 µm, 50 mm × 4.6 mm; Agilent Technologies).

2.6. RNA Extraction, Expression Analysis and Gene Copy Number Determination

Total RNA from 50 mg lyophilized plant material was extracted using the Plant RNA Isolation Kit (Stratec, London, ON, Canada) but also subjected to DNA digestion step [RNase Free DNase Set (Qiagen, Hilden, Germany)], and RNA integrity was checked on an agarose gel. Same amounts of total RNA, template cDNA for subsequent PCR reactions

was generated using Superscript™ III (Invitrogen, Waltham, MA, USA). The absence of genomic DNA contamination was confirmed using an equivalent amount of the total RNA for each sample without reverse transcription. A no-template control was included for each primer pair. Quantitative real-time PCR was performed with SsoAdvanced Universal SYBR Green Supermix (BIO-RAD, Hercules, CA, USA), with 0.5 µM of each primer. Relative RNA levels were calibrated and normalized to the level of the housekeeping gene actin. Sequence alignments of published cDNAs from the NCBI database (http://www.ncbi.nlm.nih.gov/, accessed on 15 January 2018) were used to design primers for gene isolation and specific primer pairs were designed based on the conserved regions of cDNAs. Primer sequences for Actin (Act), Limonene synthases (*Lim s*), and Pulegone reductase (*Pr*) are shown in Table 1 [20]. PCR was performed using a CFX Connect Real-Time PCR System (BIO-RAD). The thermal cycling conditions were 5 min at 95 °C, followed by 40 cycles of 15 s at 95 °C, 15 s at 58–62 °C, and 30 s at 72 °C. Transcript abundance was normalized to the transcript abundance of the actin. The double-delta CT (ΔΔCT) method was used for data analysis [21]. All reactions were run in duplicate, and average values were used in the analysis.

Table 1. Primer sequences for RT-PCR.

Gene	*Forward Primer Sequence (5′-3′)*	*Reverse Primer Sequence (5′-3′)*
Act	GCTCCAAGGGCTGTGTTCC	TCTTTCTGTCCCATGCCAAC
LimS	TTGTGGCGAATTCTCTCGCT	GGCTTCTGAGCTGGTCACTT
Pr	GCATGGAGATCCCAGATGGC	AGTAGAGCCAGGAAGGATGGA

2.7. Statistical Analyses

Normality and homoscedasticity of the data were first checked using Shapiro–Wilk and Levene tests, respectively, Data were pooled and subjected to analysis of variance (ANOVA) means were considered significant for p values < 0.05 followed by comparison of multiple treatment levels with controls using Fisher's post hoc LSD (least significant difference) test. The significance level for all calculations was set at p values < 0.05. The purpose of PCA was to extract and display relationships among factors in the multivariate data set (stress and inoculation conditions, monoterpene synthesis structures, EO yield and total emission. The Infostat software program, version 2011 (Group Infostat, Universidad Nacional de Córdoba, Córdoba, Argentina) was used for all statistical analyses.

3. Results

3.1. Essential Oil Content and Main Constituents

In the present study, a positive impact of direct inoculation with the PGPR on EOs yield in peppermint plants was recorded. This increase was between four and fives times higher ($p < 0.05$) compared to non- inoculated control plants (Figure 1). When the plants were subjected to both MS and SS stress, the total EO content was three times higher in relation to control plants with regular irrigation ($p < 0.05$). Regarding the combination of effects, stress + PGPR, a non-significant difference was observed in relation to inoculated or non- inoculated stressed plants ($p > 0.05$) regardless of the severity of the stress applied.

Regarding, the main EO components, the concentration of menthone, menthol and pulegone were increased by the different treatments applied ($p < 0.05$) (Table 2), while the content of limonene and menthofuran were not modified ($p > 0.05$). Menthone content increased approximately 4-fold in inoculated plants and 3-fold in stressed plants compared to control plants ($p < 0.05$). When the plants were subjected to stress and inoculated, no significant differences were recorded with their respective controls (non inoculated stressed plants) ($p > 0.05$). On the other hand, the menthol content increased approximately six times in the inoculated plants in relation to control ($p < 0.05$), when the plants were grown under stress, they did not show a statistical difference despite the increment observed, in comparison with regular watering control plants ($p > 0.05$). However, in the plants

subjected to the combination of treatments (MS + PGPR), the menthol levels increased in relation to the MS non-inoculated control plants ($p < 0.05$). On the contrary, inoculation with PGPR on SS plants did not produce significant changes in relation to plants subjected to non-inoculated SS plants ($p > 0.05$) (Table 2). The pulegone content registered the same trend; an increase between 3–4.5 times on inoculated or stressed plants with respect to the control ($p < 0.05$). When the inoculated plants were subjected to stress, regardless of the strain they did not show differences with their respective controls (MS or SS) ($p > 0.05$).

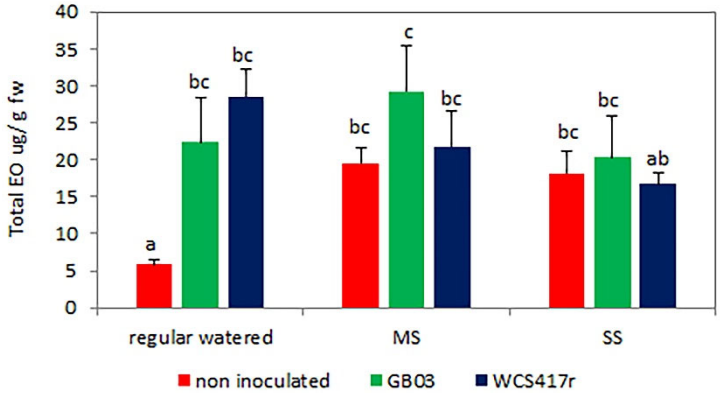

Figure 1. Essential oil yield in *M. piperita* plants grown under drought stress (MS: moderate stress, SS: severe stress) and inoculated with different Plant Growth Promoting Rhizobacteria (PGPR) strains (mean ± SE). Letters above bars indicate significant differences according to Fisher's LSD test (n = 15–20, $p < 0.05$).

Table 2. Concentrations of major Essential Oil (EO) components (μg/g fresh weight) in *Mentha piperita* plants grown under drought stress (MS: moderate stress, SS: severe stress) and inoculated with different plant growth promoting rizobacteria (PGPR) strains (mean ± SE). Means followed by the same letter in a given column are not significantly different according to Fisher's LSD test (n = 15–20, $p < 0.05$).

Treatment	Limonene	Menthone	Menthofurane	Menthol	Pulegone
Regularly watered	0.57 ± 0.10 a	0.48 ± 0.09 a	0.33 ± 0.09 a	0.41 ± 0.10 a	3.89 ± 0.39 a
GB03	1.17 ± 0.13 a	1.83 ± 0.28 b	0.65 ± 0.19 a	2.40 ± 0.66 bc	12.51 ± 2.26 b
WCS417r	1.35 ± 0.36 a	2.01 ± 0.46 b	0.67 ± 0.29 a	1.85 ± 0.17 bc	18.93 ± 2.53 b
MS					
Non-inoculated	1.45 ± 0.27 a	1.64 ± 0.22 b	0.75 ± 0.24 a	1.03 ± 0.46 ab	12.36 ± 1.52 b
GB03	1.79 ± 0.42 a	1.99 ± 0.41 b	0.35 ± 0.12 a	2.67 ± 0.40 c	17.74 ± 4.44 b
WCS417r	1.02 ± 0.22 a	2.12 ± 0.27 b	0.43 ± 0.14 a	2.34 ± 1.06 bc	13.01 ± 2.50 b
SS					
Non-inoculated	1.53 ± 0.41 a	1.49 ± 0.30 b	0.74 ± 0.29 a	1.12 ± 0.22 abc	13.31 ± 2.46 b
GB03	0.92 ± 0.19 a	1.53 ± 0.25 b	0.38 ± 0.12 a	1.62 ± 0.36 abc	11.76 ± 3.64 b
WCS417r	1.31 ± 0.26 a	1.55 ± 0.24 b	0.36 ± 0.07 a	1.47 ± 0.58 abc	10.48 ± 1.71 b

3.2. VOC Emissions

The VOC emissions showed a tendency to rise in *M. piperita* plants inoculated with WCS417r and to decrease under drought stress (Figure 2). Inoculation with WCS417r induced a 45% increase in the emissions in relation to the control plants ($p < 0.05$). In the particular case of the MS plants, although the emission of VOCs decreased by 15%, there was no statistical difference with the plants with regular irrigation ($p > 0.05$), and in the case of SS, the decrease in the emission of monoterpenes represented 50% less than control ($p < 0.05$) (Figure 2). The inoculation of plants subjected to water stress did not reveal any difference with the controls (MS or SS) ($p > 0.05$).

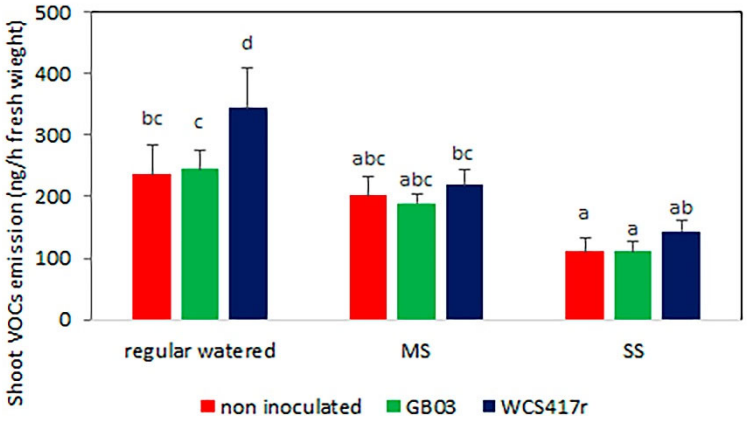

Figure 2. Variations in VOC emissions from peppermint plants subjected to moderate (MS) or severe drought stress (SS) and inoculated with different PGPR strains (mean ± SE). Different letters indicate significant differences between treatments based on Fisher's LSD test (n = 15–20, p <0.05).

3.3. Trichome Density

EOs are produced and stored in particular histological units called glandular trichomes. Glandular trichomes were recorded on both sides of the leaf, but were more numerous on the abaxial side (Table 3), with the density of peltate (PT) and capitate (CT) being approximately 2–2.5 times higher, respectively, on the abaxial face, in all the conditions evaluated (Table 3). In fact, PT are registered to be the primary places where EOs are synthesized (Turner et al., 2000).

Table 3. Glandular trichome density from peppermint subjected to moderate (MS) or severe drought stress (SS) drought stress and inoculated with different PGPR strains (mean ± SE). Means followed by different letters indicate significant differences between treatments based on Fisher's LSD test (n = 15–20, $p < 0.05$).

Treatment	Adaxial Face		Abaxial Face	
	Peltate (n per mm^2)	Capitate (n per mm^2)	Peltate (n per mm^2)	Capitate (n per mm^2)
Regularly watered	1.31± 0.09 a	4.89 ± 0.24 a	2.70 ± 0.21 a	11.83 ± 0.69 a
GB03	1.65 ± 0.13 ab	5.31 ± 0.30 a	4.17 ± 0.24b cd	12.90 ± 0.43 ab
WCS417r	2.07 ± 0.16 c	6.46 ± 0.35 b	5.43 ± 0.34 e	16.65 ± 0.58 c
MS				
Non-inoculated	1.66 ± 0.13 ab	7.06 ± 0.44 b	4.20± 0.23 cd	17.77 ± 0.68 c
GB03	1.97 ± 0.14 bc	6.55± 0.41 b	5.18 ± 0.18 de	16.87 ± 0.65 c
WCS417r	1.70 ± 0.14 b	5.24 ± 0.28 a	3.93 ± 0.20 bc	14.12 ± 0.62 b
SS				
Non-inoculated	1.61 ± 0.14 ab	6.94 ± 0.41 b	3.82 ± 0.26 bc	17.05± 0.91 c
GB03	1.79 ± 0.12 bc	6.68 ± 0.37 b	4.08 ± 0.29 bc	16.61± 0.86 c
WCS417r	1.57 ± 0.09 ab	5.14 ± 0.29 a	3.39 ± 0.29 ab	13.22 ± 0.57ab

Peppermint plants inoculated with GB03 or WCS417r showed a higher density of PT of approximately 70% on both sides of the leaf in relation to control plants. When subjected to stress, these increases fell to 50% on the abaxial and 20% on the adaxial side ($p < 0.05$). In inoculated plants subjected to stress, they did not show significant differences with the corresponding controls (MS or SS) on either side of the leaf ($p > 0.05$) (Table 3). In relation to the CT density, the response in general was similar to that observed for the PTs, except that on both faces of the leaf these values decreased by 25% in relation to their respective controls ($p < 0.05$) for plants stressed (MS and SS) and inoculated with WCS417r.

3.4. Endogenous Phytohormones

Hormones have an important function in plants that grow under abiotic stress by triggering responses that favor plants to subsist in stressful conditions, which may result in reduced growth as the plant redirects its resources to tolerate stress [22]. The levels of endogenous phytohormones in plants grown under the different treatments were affected. Inoculated plants with regular irrigation increased by 50 and 70% the ABA levels for GB03 and WCS417r, respectively, compared to non-inoculated control plants ($p < 0.05$) (Figure 3A). When plants were grown under water stress, they showed a 2.5-fold increase for both MS and SS compared to control plants (regular irrigation) ($p < 0.05$). In contrast, the plants under stress and inoculated did not reveal any changes in the ABA content in relation to controls (MS and SS) ($p > 0.05$), with the only exception being inoculation with GB03 in cultivated plants under MS, which resulted in an increase of 50% in the ABA level ($p < 0.05$) (Figure 3A). In relation to the SA levels, its content in inoculated plants increased 4 times with GB03 and was 10 times higher with WCS417r, in relation to the control of regularly watered plants ($p < 0.05$). In contrast, there was no variation in the SA concentration in plants subjected to water stress ($p > 0.05$) (Figure 3B). However, SA levels in plants inoculated with GB03 or WCS417r and subjected to MS raised by 13.5 and 15 times, respectively, in relation to the MS control ($p < 0.05$). This same trend was registered in inoculated plants subjected to SS, with an increase of approximately 10 times being recorded in relation to the SS control ($p < 0.05$) (Figure 3B).

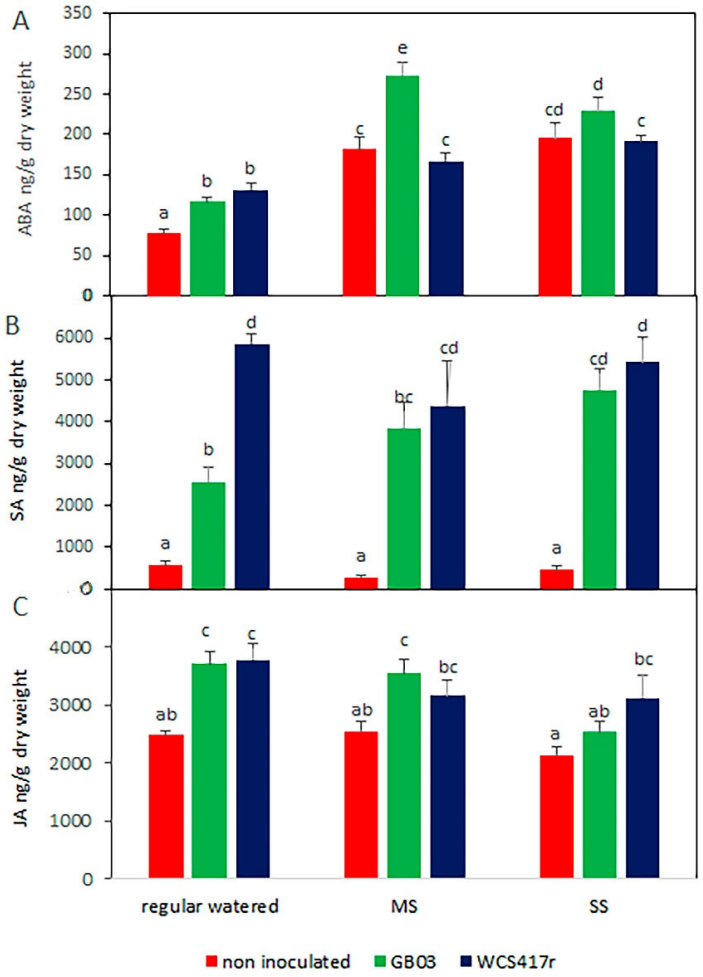

Figure 3. Endogenous plant hormone level (**A**) abscisic acid (ABA) (**B**) salicylic acid (SA) (**C**) jasmonic acid (JA) in peppermint plants inoculated with PGPR strains subjected to moderate (MS) or severe drought stress (SS). Different letters indicate significant differences between treatments based on Fisher's LSD test (n = 15–20, $p < 0.05$).

The JA endogenous concentration showed a 50% increase for both strains compared to non-inoculated plants ($p < 0.05$) (Figure 3C). Nevertheless, when plants were grown under different stress levels (MS or SS), no variation were observed in the JA level in relation to control plants irrigated regularly ($p > 0.05$). Inoculated plants cultivated under stress did not show any variation in comparison with their respective MS or SS controls, with the exception being MS plants inoculated with GB03, which demonstrated an increase approximately of 35% in relation to the MS control ($p < 0.05$) and with the WCS417r strain significantly increasing the JA level by 50% in relation to the SS control ($p < 0.05$).

3.5. Gene Expression of Key Enzymes of EO Main Components

The different treatments evaluated, of inoculation, drought stress and their combinations, affected the amount of EOs in *M. piperita*. Therefore, to achieve a deep understanding of their effects on the synthesis of major monoterpenes, the relative gene expression of the enzyme *Lim s* and *Pr* was analyzed by q-PCR (Figure 4). The main compounds presented in the EOs of the peppermint plants used in the present study are menthol, menthone, limonene, menthofuran and pulegone. In the first step of monoterpene biosynthesis in the Mentha species, the enzyme *Lim s* is involved [23], which is responsible for the cyclization process of geranyl diphosphate (GPP) to form limonene [24]. Then, limonene is transformed into pulegone through several reactions [25]. The *Pr* enzyme was previously found to reduce pulegone to menthone or isomenthone in a 2.5:1 ratio [26,27]. In the current study, we considered the *Lim s* gene, the first gene responsible for the synthesis of the monoterpenes, and also Pr, which is particularly important for reducing pulegone (and a major component in our plants).

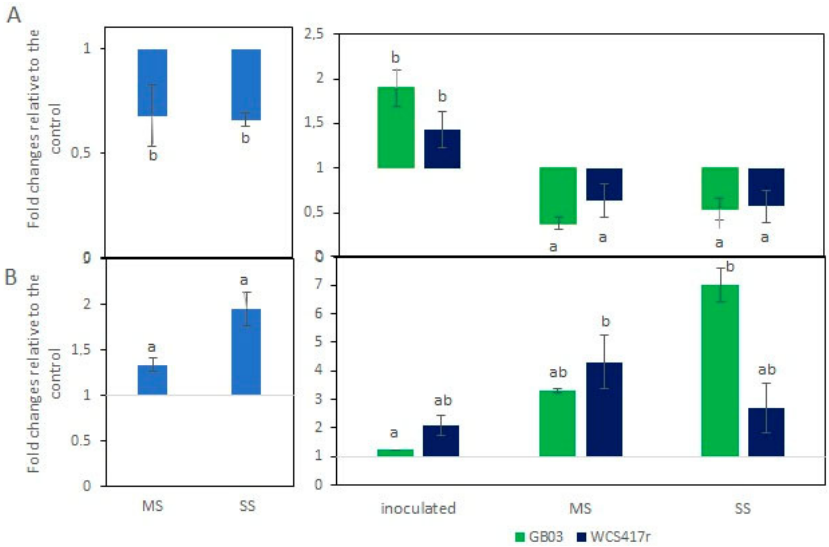

Figure 4. Limonene synthase (*Lim s*) (**A**) and pulegone reductase (*Pr*) (**B**) gene expression of peppermint plants subjected to moderate (MS) or severe drought stress (SS) and inoculated with different PGPR strains (mean ± SE). Values are fold changes relative to the control. Different letters indicate significant differences between treatments based on Fisher's LSD test (n = 5, $p < 0.05$).

The expression of *Lim s* was downregulated in plants subjected to drought stress (Figure 4A). In contrast, the inoculation of regularly irrigated plants affected it positively, with GB03 being overexpressed by 2-fold more than the control ($p < 0.05$), while the rest of the treatments did not present differences with the control ($p > 0.05$). In relation to the transcription of the gene that codes for the enzyme *Pr* (Figure 4B), positive alterations were observed in the different treatments carried out, with drought stress MS and SS upregulating *Pr* expression at the same level as regularly irrigated inoculated plants, but with these values not being statistically different ($p > 0.05$) (Figure 4B). MS plants inoculated with GB03 and WCS417r showed an upregulation in *Pr* expression of 3- and

4-fold, respectively. *Pr* in inoculated SS plants was upregulated for both evaluated strains, but was markedly stimulated for GB03 by almost 7-fold ($p < 0.05$), which was the highest expression of *Pr* observed in the present study.

3.6. Principal Components Analysis (PCA)

In order to relate the treatments (stress and inoculation conditions) to the factors evaluated (PT, EO yield, VOCs emission, ABA, JA, SA), a multivariate technique for analyzing quantitative data (PCA) was used. This type of analysis provides a plot that simplifies the perception and interpretation of the data set and the variables. In the PCA (Figure 5) for the different stress and inoculation strains, CP1 explained 54.8% of the variability of the data, while CP2 accounted for 29.7%. Taken together, both axes explained 84.5% from the variations in the data, with a cophenetic correlation coefficient of 0.988. We registered a robust positive correlation between PT, EOs, SA and JA, but in contrast, ABA and VOC emission had a weak relation with these factors mentioned above. The plot shows that MS as well as inoculation treatment (GB03 or WCS) or their combination (MS + PGPR) were positioned near to all the variables assessed (green ellipse), revealing a strong effect with respect to PT, EO and its emission as VOCs. In contrast, control and SS treatments, alone or combined with GB03 or WCS, were located far from the variable assessed (yellow ellipse), representing a minor effect.

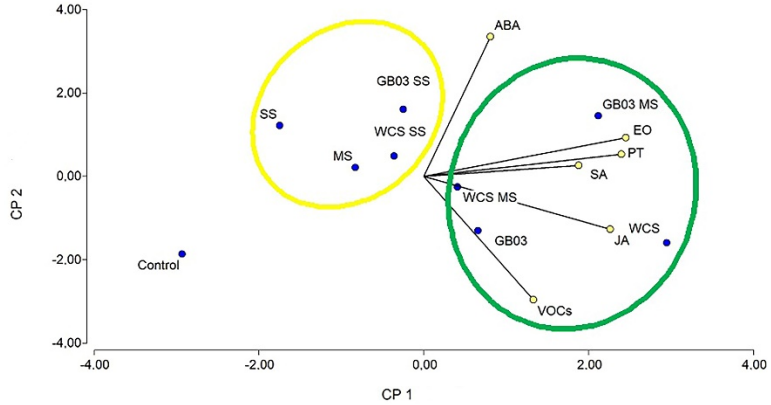

Figure 5. PCA plot for the response of peppermint plants subjected to drought stress inoculated with PGPR strains. Green ellipse, drawn manually, shows a strong effect of MS as well as inoculation treatment (GB03 or WCS417r) or their combination (MS + PGPR) with the variable PT, EO and its emission as VOCs. In contrast, control and SS treatments, alone or combined with GB03 or WCS417r, were located far from the variable assessed (yellow ellipse), representing a minor effect. EO: Essential Oil, VOC: Volatile Organic Compounds, PT: Peltate Trichome; JA: Jasmonic Acid; SA: Salicylic Acid and ABA: Abscisic acid; MS: moderate stress; SS: severe stress.

4. Discussion

In the present study, we have validated that under regular irrigation GB03 and WCS417r strains enhance trichome density and EO yield on peppermint plants, as earlier reported by Cappellari et al., [17]. In previous studies it was observed that peppermint plants inoculated with GB03 or WCS417r and grown under drought stress showed a significant increase in shoot fresh weight with respect to MS and SS plants, which was approximately 15% higher than the respective control (MS or SS), with the same tendency being observed for leaf area and number of leaves. MS-inoculated plants showed an increase in shoot fresh weight compared to non-inoculated plants [14].

In the current research, when plants were grown under water stress, the EO production showed an increase, at similar levels as in inoculated plants, but the inoculation of stressed plants did not modify this parameter. As expected, PT revealed the same tendency as EO yield. In concordance with our results, Askary et al., [28] observed that the number of glandular trichomes were increased under salt stress in *M. piperita*. In addition, there was

a strong correlation previously reported between EO production and the total number of glandular trichomes [29,30]. It is worth mentioning that when aromatic plants are cultivated in semi-arid environments they are mostly more "fragrant" than when cultivated in moderate climates. In addition, under stressed environments, the amounts of EO are increased [1]. According to these considerations, the more severe the stressed the condition, the more severely it will influence the metabolism, having an effect on the biosynthetic pathways involved in the synthesis of secondary metabolites [1,31].

It has been suggested that the induction of EO yield under drought stress may be because when plants grow under stress conditions, they only allocate low amounts of carbohydrates from photosynthesis to plant development, and instead use these for the synthesis of secondary and reserve metabolites, thus generating a balance between growth and defense [1,32,33]. The increase in EO in this case could be working as a mechanism to dissipate "extra" energy [1,34]. Indeed, in favorable conditions, most of the plant's energy is directed to primary metabolism and only a small portion to the secondary metabolism [35,36]. Although the system achieves a proper function under normal environmental conditions, the presence of water stress causes an imbalance, and when water is restricted, the stomata are closed. Consequently, significantly fewer reducing equivalents (NADPH + H+) are consumed to fix CO_2 by the Calvin cycle. As a consequence, only minor amounts of oxidized reduction equivalents (NADP+) are accessible as electron acceptors. Generating an accumulation of a large number of NADPH + H+ produces a strong over-reduced state in the chloroplasts, and consequently, the photosystems will favor the production of oxygen, generating superoxidized radicals that must be eliminated by the antioxidants enzymes. Furthermore, due to the abundance of reduced coenzymes, all reactions that use NADPH + H+ will be favored, for example the synthesis of highly reduced secondary metabolites [1,37], including EOs, as observed in the present study. This increment in EO yield was connected with a rise in PT density, where the EOs are synthesized and stored and by the upregulation observed in the expression of *Pr* in plants (inoculated or not) grown under drought stress. Although we do not observe any up regulation of *Lim s*, it has been reported that in peppermint, the most important enzymes involved in the monoterpene synthesis, were controlled at the gene expression level [38]. The difference shown between substance produced and transcript levels may be explained by either post-transcriptional modification, protein stability or an increased flux in the metabolic pathway [39].

It is important to highlight the inconsistencies found in the bibliography in relation to water stress and EO yield, even in plants of the same species. In agreement with our observations, Zade et al. [40] registered two fold more EO in peppermint grown under water stress. In concordance, in *S. officinalis*, MS generated a 4.5-fold increment in EO content and a 2.6-fold increment in plants subjected to SS [32]. In pennyroyal plants grown under water stress, a 40% increase in EO content was also shown [28], with comparable responses having been observed for other Lamiaceae species grown under water stress, such as *Lavandula angustifolia* [41], *Petroselinum crispum* [42] and *Thymus vulgaris* [43]. However, in a one-year field trial, García-Caparrós et al. [44] observed that different Lamiaceae subjected to drought stress did not present variations in the amount of EOs, while in *Lavandula latifolia* and *Salvia sclarea*, the EO content actually decreased. Other authors have also indicated negative effects on EO content due to water stress in *Menhta arvensis* [45], ref. [46] *Rosmarinus officinalis* and *Salvia officinalis* [47].

In relation to the main monoterpenes (menthone, menthol and pulegone), water stress also induced changes in EO concentrations. In agreement with our observations, Zade et al. [40] recorded a significant increase in menthol content in *M. piperita* plants subject to water stress, with similar results being obtained in M. arvensis grown under water stress [45]. Related to this, it was reported that oxygenated monoterpenes, for example menthone, menthol and pulegone, could be reason of the EOs antioxidant activity [48].

Many of the differences observed in the bibliography related to the EO content may be due to variations in the experiment design. In general, there are difference in the substrate used and in the length of the assays. For example, Zade et al., [40] used a

mixture of soil, peat and coco peat, while Arpanahi et al. [49] used enriched soil and Asghari et al., [28] worked with sandy soil. In contrast, our assay was carried out in vermiculite, irrigated with Hoagland's solution in order to control the supply of nutrients. Another distinctive methodology difference is concerning the generation of water stress and the irrigation method applied. Zade et al. [40] used drinking water and the stressed plants were rehydrated, since they suspended irrigation for 2 rounds and then resumed it again. Regarding VOCs, the results obtained in the current study showed that water stress reduced the emissions, which were lower for higher applied stresses. Comparable results were recorded in *Salvia dolomitica* [50] and in woody plants [51]. In contrast, in other studies, drought stress did not influence the emission of VOCs, such as in *Zataria multiflora* [52]. Nevertheless, it was reported that water stress stimulated emission in thyme [53]. The diversity of results about the consequence of water stress on plant VOC emissions could also be due to differences in the degree of stress or damage produced to the plant by drought. Therefore, a high level of drought could reduce emissions, whereas a minor drought stress could actually increment emissions [54]. Nevertheless, a rise in the emissions of different terpenes can in some cases be a result of an increase in trichome density [55]. It should be emphasized that in stressed plants in the present study, we observed an increase in EOs but not in VOC emissions. Studies carried out on other plant species have demonstrated comparable variations in volatile emissions vs. internal oil [56,57]. In aromatic plants, healthy plants maintain a basal emission of volatiles, which are released from the glandular trichomes where they are accumulated [4]. Initially, it was believed that the volatiles simply diffuse through cell membranes because of the lipophilic properties of these metabolites. However, current studies indicate that for several plant volatiles compounds, the internal concentration required for them to passively diffuse is very high, suggesting that this amount could be toxic within the membranes themselves [30,58,59]. Just exactly how these metabolites are released into the atmosphere is not known, but several hypotheses have been developed, among which the presence of an ATP-driven membrane transporter has been suggested, whose function is to expel toxic compounds from the plant itself, thereby avoiding their accumulation [58–60]. Active transport proteins could also allow strict controls on VOC emission rates, which might have allowed plants to send volatile-encoded messages with a higher accuracy [58].

Currently, there is brief evidence available on the consequences of drought stress on aromatic plants inoculated with PGPR. In a previous report, we described that under similar conditions inoculation with these strains alleviated the adverse effects of water stress, resulting in an increase of shoot weight, leaf area and significantly less reduction in plant growth. Additionally, drought-stressed plants inoculated with PGPR had a remarkably higher total phenolic content and enzymatic activity than water-stressed plants without PGPR inoculation. The peroxidation of membrane lipid was also reduced in PGPR treated plants subjected to water stress [14].

Is well known that phytohormones have an important function in plant growth, immunity and the accumulation of secondary metabolites. In particular, JA is implicated in stimulating trichomes formation, as well as the biosynthesis of EOs. However, it does not act alone but instead interacts with SA, thus co-regulating the synthesis of secondary metabolites [20,56]. Regarding the effect observed in the current study, the increase in PT and EO yield might be related to the increase in JA and SA recorded in plants subjected to stress and PGPR inoculation (Figure 5). In fact, the levels of JA and SA increased in inoculated plants subjected to water stress at similar levels, as happened in plants only inoculated. It was reported previously that JA and SA are implicated in the ISR signaling pathways triggered by PGPR [12,20]. ABA also showed the same tendency, with, as expected, levels increasing in relation to regularly watered plants, since ABA controls the expression of several genes that help the plant to survive under drought stress [61]. However, the levels found in plants stressed and inoculated were similar to those in plants subjected to water stress.

In the present study, as shown in the scheme (Figure 6), there was a rise in endogenous phytohormone levels (SA, JA, ABA), as well as an increase of EO yield and PT density, in peppermint treated with PGPR and subjected to water stress compared to regularly watered plants. In contrast, VOC emissions were reduced. The same positive effect was observed in trichome density, EO main components and total EO yield in inoculated PGPR stressed plants, and also in plants only inoculated or MS stressed alone, as can be clearly seen in the PCA graphic (green ellipse) (Figure 5).

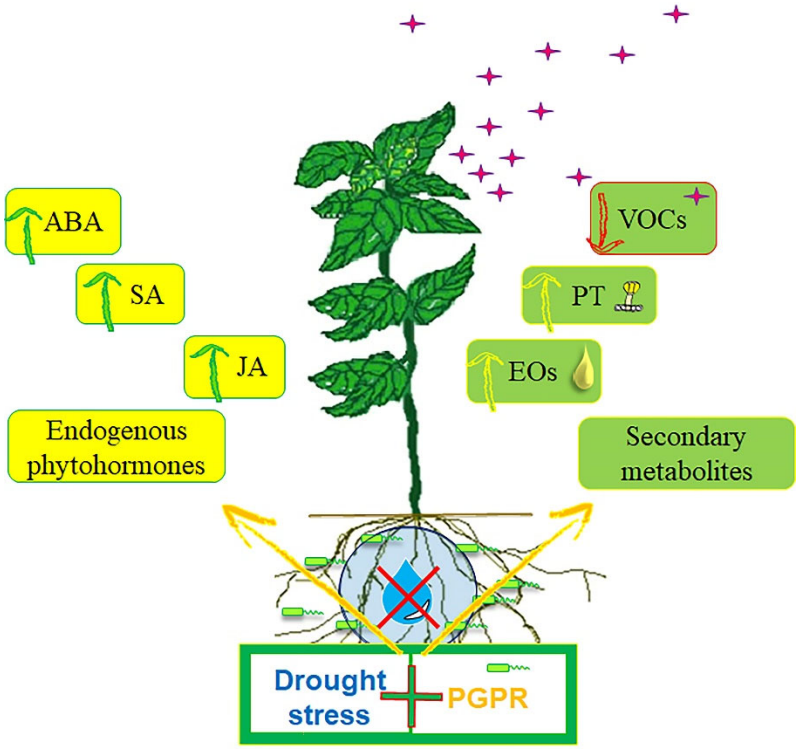

Figure 6. Schematic plant responses involved in *Mentha piperita* subjected to drought stress and treated with PGPR. Under these conditions, a rise in endogenous phytohormone levels (SA, JA, ABA) was observed as well as an increment in EO yield and PT density compared to regularly watered plants. In contrast, VOC emissions were reduced. EO: Essential Oil, VOC: Volatile Organic Compounds, PT: Peltate Trichome; JA: Jasmonic Acid; SA: Salicylic Acid and ABA: Abscisic acid; PGPR: plant growth promoting rhizobacteria.

Summing up, it has been shown that drought stress or inoculation per se are capable of inducing an increase in EO yield and PT density, suggesting that these treatments when used individually seem to reach a limit, in relation to the kind of plant defense response, and with no synergism effects being observed with the application of both treatments together. In contrast, a synergistic effect of both treatments was described on total phenolic compounds by Chiappero et al., [14].

5. Conclusions

It is clear that water deficit is a significant stress that reduces plant yield and is associated with a reduction of photosynthesis, and consequently, of plant development. Therefore, water deficiency can have a significant effect on a plant's whole metabolism; secondary metabolite synthesis is also altered. A better understanding of the adaptive strategy of plants grown under water stress is important from an economic and ecological perspective, particularly concerning aromatic plant production. In the current investigation, it was shown that there was an increase in the production of EOs in inoculated or uninoculated peppermint, under drought stress. The results from the present study, taken together with those from a previous investigation [14] have opened up important possibilities for

improving aromatic plant productivity, particularly in the case of peppermint. The results confirm the importance of the concept of handling drought stress with the intention of enhancing aromatic plant productive capacity by the inoculation with beneficial rhizobacteria. When the purpose of the crops is to obtain *M. piperita* plants with a high yield of secondary metabolites, EOs and phenolic compounds, we can suggest appropriate inoculation with strains WCS417r or GB03 and the generation of moderate drought stress. Yet, if the objective is greater biomass production, inoculation with PGPR is highly recommended with regular irrigation and avoiding cultivation under drought stress. However, it is crucial to carry out field trials with a rigorous and controlled design, before considering the induction of water deficit in the crop of peppermint plants inoculated with PGPR micro-organisms.

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Abbreviations

ABA: abscisic acid, CT: capitate trichome, EOs: essential oils, FC: field capacity, JA: Jasmonic acid, Lim s: limonene synthase; MS: moderate stress, PGPR: plant growth promoting rhizobacteria, Pr: pulegone reductase; PT: peltate trichome SS: severe stress, VOC: volatile organic compounds, ROS: reactive oxygen species; SA: salicylic acid.

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