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Volatile Organic Compounds from Rhizobacteria Increase the Biosynthesis of Secondary Metabolites and Improve the Antioxidant Status in *Mentha piperita* L. Grown under Salt Stress

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Abstract: Salinity is a major abiotic stress factor that affects crops and has an adverse effect on plant growth. In recent years, there has been increasing evidence that microbial volatile organic compounds (mVOC) play a significant role in microorganism–plant interactions. In the present study, we evaluated the impact of microbial volatile organic compounds (mVOC) emitted by *Bacillus amyloliquefaciens* GB03 on the biosynthesis of secondary metabolites and the antioxidant status in *Mentha piperita* L. grown under 0, 75 and 100 mM NaCl. Seedlings were exposed to mVOCs, avoiding physical contact with the bacteria, and an increase in NaCl levels produced a reduction in essential oil (EO) yield. Nevertheless, these undesirable effects were mitigated in seedlings treated with mVOCs, resulting in an approximately a six-fold increase with respect to plants not exposed to mVOCs, regardless of the severity of the salt stress. The main components of the EOs, menthone, menthol, and pulegone, showed the same tendency. Total phenolic compound (TPC) levels increased in salt-stressed plants but were higher in those exposed to mVOCs than in stressed plants without mVOC exposure. To evaluate the effect of mVOCs on the antioxidant status from salt-stressed plants, the membrane lipid peroxidation was analyzed. Peppermint seedlings cultivated under salt stress and treated with mVOC showed a reduction in malondialdehyde (MDA) levels, which is considered to be an indicator of lipid peroxidation and membrane damage, and had an increased antioxidant capacity in terms of DPPH (2,2-diphenyl−1-picrylhydrazyl) radical scavenging activity in relation to plants cultivated under salt stress but not treated with mVOCs. These results are important as they demonstrate the potential of mVOCs to diminish the adverse effects of salt stress.

Keywords: mVOCs; Plant growth promoting rhizobacteria; PGPR; *Mentha piperita*; *Bacillus amyloliquefaciens* GB03; salt stress; secondary metabolites; MDA; DPPH

1. Introduction

Many aromatic plants, such as *Mentha piperita* L. (peppermint), are important sources of essential oil (EO) production. The EOs are generated and stored in glandular trichomes, where they form complex mixtures of secondary metabolites (SM) mainly composed of the volatile mono- and sesquiterpenes responsible for the characteristic aromas of various plant species [1,2]. Therefore, the quality of aromatic plants is recognized by the composition and concentration of these components for each species. Furthermore, the quantity and quality of SM is determined by environmental factors including temperature, soil quality, light intensity, and/or water availability [3].

Biotic and abiotic stresses are major constraints on crop yield, with environmental stress representing a strong restriction on increasing crop productivity as well as affecting the use of natural resources. A soil is considered to be saline when the ion concentration reaches an electrical conductivity of >4 dS m^{-1}, measured on a saturated soil at 25 °C, and consequently interferes with the growth of species of agricultural interest [4]. Salinity impacts agricultural production in most crops by affecting the physical-chemical properties of the soil and the ecological balance of the cultivated area [5]. As salinity affects many aspects of the physiology and metabolism of the plants, the presence of soluble salts in general has a negative consequence for the plant's growth by decreasing the water potential and thus restricting the absorption of water by the roots (osmotic effect). In addition, the absorption of specific saline ions leads to their accumulation in tissues in concentrations at which they can become toxic and induce physiological disorders (ionic toxicity) in the plant, with high concentrations of saline ions being able to modify the absorption of essential nutrients and leading to nutritional imbalances (nutritional effect) [6]. These effects are reflected by a decrease in germination, vegetative growth, and reproductive development [4,7].

Plant tolerance to salt stress is linked to the use of different strategies, including osmotic adjustment, the exclusion of toxic ions from the aerial part, translocation of photoassimilates to underground organs, an increased growth of the root system, and ensuring the availability of water and nutrients, among others. Furthermore, salinity can produce an accumulation of reactive oxygen species (ROS) [6], which may lead to a deterioration of photosynthetic pigments, lipid peroxidation, alterations in the selective permeability of the cell membranes, protein denaturation, and DNA mutations [8–10]. Damage of the cell membrane produces small hydrocarbons such as malondialdehyde (MDA), which is a sign of membrane cellular damage. Plants have well-described protection and repair systems that mitigate ROS damage. In addition, certain species have developed protective mechanisms that include enzymatic and non-enzymatic components [11,12].

Plant growth promoting rhizobacteria (PGPR) are beneficial microorganisms capable of colonizing the rhizosphere of plants and benefiting them both directly and indirectly [13]. It is well known that PGPR functions in different ways: synthesizing specific compounds for the plants, helping the uptake of nutrients, and protecting the plants from diseases [14–16]. In general, it has been observed that the negative effects that salinity produces in plant development can be mitigated by the use of microorganisms as inoculants, which is an alternative technology to improve the abiotic stress tolerance capacity of plants [17–21]. In this regard, considerable attention has been focused on understanding the molecular, physiological, and morphological mechanisms underlying rhizobacterial-mediated stress tolerance. In fact, the mechanisms by which these bacteria mediate abiotic stress tolerance continue to be widely studied, largely because they are difficult to elucidate [22,23].

Advances in research have revealed that certain PGPR strains are capable of emitting microbial volatile organic compounds (mVOCs) [24–28]. These compounds mainly consist of an abundant and very complex mixture of compounds, including alcohols, alkanes, alkenes, esters, ketones, sulfur, and terpenoids, characterized by their low molecular weight and high vapor pressure under normal conditions, which can vaporize significantly and enter the atmosphere. The analysis of mVOCs is a developing research area that has an effect on the applied agricultural, medical, and biotechnical applications, with a related interesting mVOC database containing available information regarding microbial volatiles having been published [29]. Recent studies have also provided new insights into the participation of mVOCs in inter- and intra-specific communication [30]. These compounds have been observed to have the ability to promote plant growth and induce systemic resistance (ISR) against pathogenic organisms, thereby improving the well-being of crops [24,27,28,31,32]. VOCs from *Paraburkholderia phytofirmans* have been shown to increase plant growth rate and tolerance to salinity, reproducing the effects of direct bacterial inoculation of roots [32]. Thus, the emission of mVOCs is currently recognized as being a very relevant aspect in microorganism–plant interactions [17,21,28,33,34].

We have previously demonstrated that both the direct inoculation of PGPR and exposure to VOCs emitted by these rhizobacteria stimulate the biosynthesis of SM and increase the biomass production

in different aromatic plants [25,26,35–39]. Although there are few reports about the effects of mVOCs emitted by rhizobacteria on the SM yield of aromatic plants under conditions of abiotic stress, studies related to the emission of volatile organic compounds with biological activity by rhizobacteria is a novel area attracting increasing interest.

It should also be noted that it is necessary to examine the use of fertilizers and chemical synthesis pesticides related to the concentration of salts in the soil in order to develop sustainable agriculture, as this is key to assessing the proposal of alternative and complementary strategies. Taking this into consideration, among the possible alternatives, the use of microbial inoculants, considered to be a clean technology aligned with the principles of sustainable agriculture, becomes more relevant. Thus, the present study was founded on the hypothesis that the investigation of mVOCs with respect to the description of their biological functions and ecological roles is crucial for elucidating the mechanisms related to the control of critical biological processes in plant health and that this could also offer useful benefits to confront agronomic and environmental complications. In this present study, the aim was to explore the potential of mVOCs in ameliorating salinity effects in *M. piperita,* with an important objective of the study being to evaluate the role of mVOCs in EOs and the phenolic compound levels, as well as their function in the antioxidant status of plants grown under salt stress conditions.

2. Materials and Methods

2.1. Bacterial Strains and In Vitro Plant Treatments

2.1.1. Bacterial Cultures

Bacillus amyloliquefaciens GB03 (originally described as *Bacillus subtilis* GB03) [40] strain was grown on LB (Luria-Bertani) medium for routine use and maintained in nutrient broth with 15% glycerol at −80 °C for storage. The bacterial culture was grown overnight at 30 °C and centrifuged at 120, washed twice in 0.9% NaCl by Eppendorf centrifugation (4300× *g*, 10 min, 4 °C), re-suspended in sterile water, and adjusted to a final concentration of ~10^9 CFU/ mL for use as an inoculum.

2.1.2. Plant Micropropagation

The *M. piperita* plant is a commercially cultivated crop grown in the Traslasierra valley (Córdoba province, Argentina). Young shoots from peppermint were surface-disinfected and micropropagated, as previously described by Santoro et al. [26].

2.1.3. In Vitro Exposure to mVOCs

Single nodes from aseptically cultured plantlets were planted in sterilized glass jars (250 mL) containing 50 mL MS (Murashige and Skoog) solid media with 0.8% (*w/v*) agar and 3% (*w/v*) sucrose. Then, a small (10 mL) glass vial containing ca. 3 mL of Hoagland media with 0.8% (*w/v*) agar and 3% (*w/v*) sucrose was introduced into each jar. The small vial was inoculated with GB03 (50 µL), which served as the source of bacterial volatiles, with sterile water used in the control. Plants were exposed to mVOCs without having any physical contact with the rhizobacteria. Jars containing plants and bacteria were covered with aluminum foil, sealed with parafilm to avoid contamination, and placed in a growth chamber under controlled conditions (16/8-h light/dark cycle), temperature (22 ± 2 °C) and relative humidity (~70%). After 30 days, all plants were collected [38].

2.1.4. Treatments

MS media (plant growth media) and Hoagland media (bacterial growth media) were supplemented with different salt concentrations: 0, 75, and 100 mM NaCl. For each experimental set, both the plant and bacteria were grown under the same concentration of NaCl but without contact with each other. Salt level concentrations were selected based on previous observations: at lower concentrations (25 and

50 mM), plant growth was not affected, and at higher levels (125 and 150 mM), the rooting capacity decreased significantly. Experiments were repeated three times (10 jars per treatment; 1 plant/jar).

2.2. Essential Oil Extraction and Analysis

Shoot samples were individually weighed and subjected to hydrodistillation in a Clevenger-like apparatus for 40 min. The volatile fraction was collected in dichloromethane, and β-pinene (1 µL in 50 µL ethanol) was added as an internal standard (as it was previously reported, β-pinene is not present in peppermint plants [37]). The major *M. piperita* EO components, which comprise ~60% of the total oil volume, are limonene, linalool, (−) menthone, (−) menthol, and (+) pulegone. These compounds were quantified in relation to the standard added during the distillation procedure described above. The flame ionization detector (FID) response factors for each compound generated essentially equivalent areas (differences $p < 0.05$).

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 were as follows: 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. The oil components ((−) menthone, (−) menthol, and (+) pulegone) were established by comparison of the diagnostic ions (NIST 2014 library) and GC retention times with those of the respective authentic standard compounds purchased from Sigma-Aldrich [34]. GC analysis was performed using a Shimadzu GC-RIA gas chromatograph fitted with a 30 m × 0.25 mm fused silica capillary column coated with Supelcowax 10 (film thickness 0.25 µm). The GC operating conditions were as follows: 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. Total Phenolic Content (TPC) Determination

The total phenolic content of the extract was determined by the Folin–Ciocalteu method, as previously described by Cappellari et al. [41]. The TPC were expressed in terms of µg gallic acid (a common reference compound) equivalent per g plant fresh weight using the standard curve.

2.4. Antioxidant Activity

The capacity of radical scavenging in extracts against stable DPPH• (2,2-diphenyl−1-picrylhydrazyl) was determined by the Brand-Williams et al. method [42] with minor modifications, as previously described by Chiappero et al. [43]. A calibration curve was obtained using ascorbic acid, and the scavenging capacity of the plant extracts was expressed as mM ascorbic acid equivalents (AAE) per g fresh weight (mM AEE/g FW). All experiments were performed in triplicate for each experimental unit.

2.5. Lipid Peroxidation

Lipid peroxidation was measured by quantifying the malondialdehyde (MDA) production using the thiobarbituric acid reaction. The MDA content was measured following the method of Heath and Packer [44], with some modifications, as reported by Chiappero et al. [43]. The amount of MDA was determined by its molar extinction coefficient (155 mM^{-1} cm^{-1}), which was expressed as µmol MDA/g FW (grams of fresh weight). The experiments were performed in triplicate for each experimental unit.

2.6. Statistical Analysis

Data were subjected to a two-way analysis of variance (ANOVA) (mVOcs × salt stress), followed by a comparison of multiple treatment levels with those of the control, using the post hoc Fisher LSD test. Infostat software version 2018 (Group Infostat, Universidad Nacional de Córdoba, Argentina) was used for the statistical analysis. Principal component analysis (PCA) using Infostat statistical package was conducted. The analysis of extracts shows the relationships among the treatments (mVOCs

exposure and salt stress conditions) and the different variables measured (EO, TPC, lipid peroxidation (MDA), and antioxidant capacity (AAE)). At least 15 observations were used for each treatment in the multivariate dataset.

3. Results

3.1. Essential Oil

Peppermint plants subjected to salt stress showed a reduction in EO content. Plants grown under 75 or 100-mM salt concentrations and those not treated with mVOCs revealed a 50% decrease in EO yield ($p < 0.05$) (Figure 1). When plants were treated with mVOCs under control conditions, the EO content rose approximately 3.3 times compared to plants not exposed to mVOCs (Figure 1). When plants were grown under salt stress conditions and treated with mVOC, positive effects of mVOCs on EO yields were detected. The levels of EOs increased approximately 5.6 and 6.5-fold in plants grown under 75 or 100 mM and treated with mVOCs, respectively, in relation to plants subjected to salt conditions but not treated with mVOCs, with a statistically significant interaction effect between salt stress and mVOCs being found ($p < 0.05$).

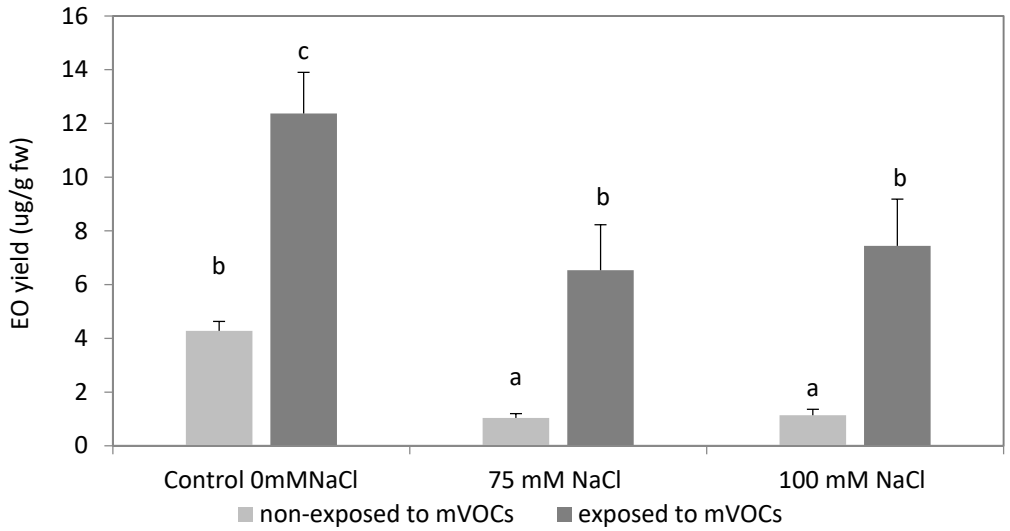

Figure 1. Essential oil yield in *Mentha piperita* plants grown under different salt concentrations (0, 75, and 100 mM NaCl) and exposed to *B. amyloliquefaciens* GB03 mVOCs (mean ± SE). Values followed by the same letter in a column are not significantly different according to Fisher's LSD test ($p < 0.05$).

Regarding the main compounds of the EOs, growing under salt stressed conditions resulted in a decrease in menthone and menthol (Table 1); although menthol content was approximately 3.5 times lower in plants grown under 75 or 100 mM concentrations and not treated with mVOCs ($p < 0.05$), the effect on menthol concentration was not statistically significant but followed the same trend as for menthone, which was significant. However, the pulegone concentration was not significantly different for control plants exposed to salt. For plants treated with mVOCs, the levels of menthone and pulegone increased approximately 2 and 3-fold, respectively, compared to those of the corresponding controls at each salinity level. However, the menthol concentration was not modified by mVOC exposure. In plants submitted to 75 mM NaCl and treated with GB03 mVOCs, the concentrations of menthone, menthol, and pulegone were approximately 6.7, 5.8, and 3.4-fold higher, respectively, in relation to plants subjected to salt conditions but not treated to mVOCs and similar to plants treated to mVOCs and not salt stressed. At 100 mM NaCl, the menthone and pulegone contents revealed the same tendency, with an increase observed in plants treated with mVOCs ($p < 0.05$), but the menthol concentration was not modified by the mVOCs (Table 1).

Table 1. Concentrations of main essential oil (EO) compounds in *Mentha piperita* grown under salt stress media (0, 75, and 100 mM NaCl) and exposed to *B. amyloliquefaciens* GB03 mVOCs emission (mean ± SE). Values are mean ± standard error (SE).

NaCl Concentration	(−)-Menthone (µg/g fw)	(−)-Menthol (µg/g fw)	(+)-Pulegone (µg/g fw)
0 mM			
control	0.99± 0.28 *b*	1.07± 0.15 *a*	1.18± 0.14 *a*
B. amyloliquefaciens GB03	2.27± 0.42 *c*	1.14± 0.23 *a*	5.29± 0.54 *c*
75 mM			
control	0.25± 0.05 *a*	0.10± 0.05 *a*	0.55± 0.12 *a*
B. amyloliquefaciens GB03	1.55± 0.17 *bc*	0.81± 0.03 *a*	2.73± 0.41 *b*
100 mM			
control	0.26± 0.05 *a*	0.22± 0.08 *a*	0.56± 0.13 *a*
B. amyloliquefaciens GB03	1.35± 0.49 *b*	0.63± 0.03 *a*	2.87± 0.79 *b*

Means followed by the same letter in a given column are not significantly different according to Fisher's LSD test ($p < 0.05$).

3.2. Total Phenolic Content

The level of TPC in plants subjected to salt stress conditions increased with the severity of the NaCl concentration ($p < 0.05$), both in plants exposed and not exposed to mVOCs. In plants grown under salt conditions (75 or 100 mM), the TPC levels rose by 15 and 50%, respectively, in relation to control plants (Figure 2). In addition, the plants subjected to both concentrations of NaC and treated with GB03 VOCs registered an increase in TPC compared to non-exposed plants ($p < 0.05$), but no statistically significant interaction effect was found ($p > 0.05$). The highest TPC concentrations were detected in plants treated with salt 100 mM and mVOCs.

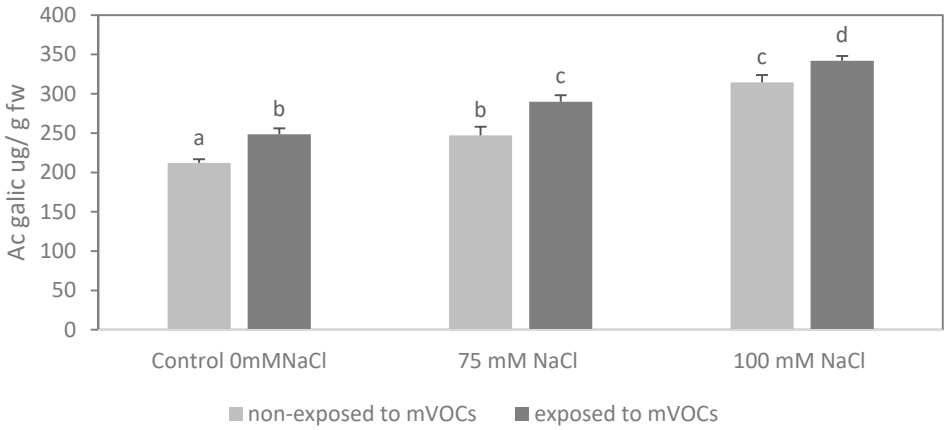

Figure 2. Total phenolic content of *Mentha piperita* plants grown under salt stress media (0, 75, and 100 mM NaCl) and exposed to *B. amyloliquefaciens* GB03 mVOCs emission (mean ± SE). Values followed by the same letter in a column are not significantly different according to Fisher's LSD test ($p < 0.05$).

3.3. Radical Scavenging Capacity

The antioxidant capacity of the DPPH• radical scavenger increased 2.6 and 3.6-fold in peppermint leaves grown under 75 and 100 mM NaCl conditions, respectively ($p < 0.05$) (Figure 3). Moreover, when plants were subjected to salt conditions and treated with mVOCs, the antioxidant capacity increased ($p < 0.05$) by 50% and 30% for 75 and 100 mM NaCl, respectively, in relation to salt stressed plants not exposed to mVOCs. The highest levels of antioxidant activity were observed when plants were exposed to VOCs and grown under 100 mM NaCl conditions, with the ascorbic acid equivalents (AAE) increasing 4.75-fold with respect to control plants (not exposed to mVOCs).

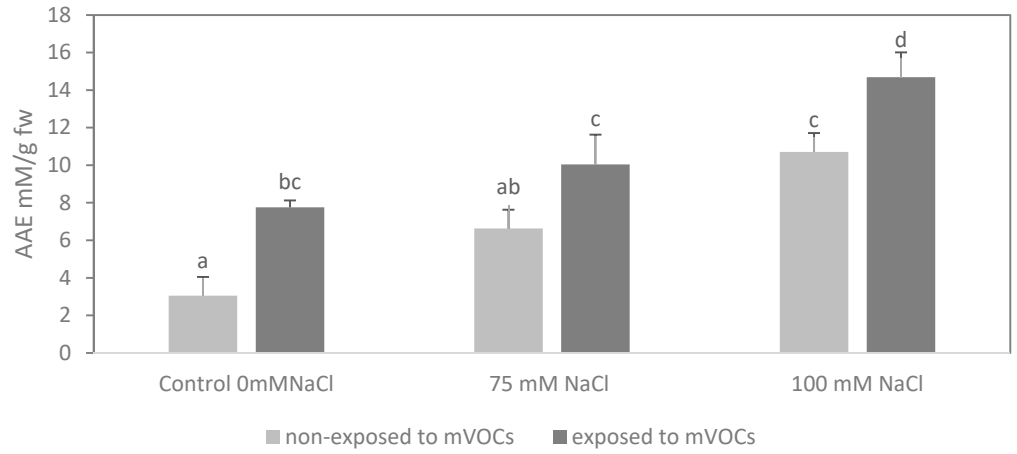

Figure 3. Antioxidant activity expressed as ascorbic acid equivalents (AAE) in *Mentha piperita* grown under salt stress media (0, 75, and 100 mM NaCl) and exposed to *B. amyloliquefaciens* GB03 mVOCs emission (mean ± SE). Values followed by the same letter in a column are not significantly different according to Fisher's LSD test ($p < 0.05$).

3.4. Lipid Peroxidation

Oxidative damage to the membrane lipids was observed due to salt stress, as shown by the MDA levels (Figure 4), with the highest MDA levels being observed ($p < 0.05$) at the higher salt concentration. The lipid peroxidation increased 1.4 and 2-fold in 75 and 100 mM NaCl treated plants, respectively, in relation to control plants. For plants treated with mVOCs and subjected to salt stress, the MDA content was approximately 25% lower than for plants stressed and not treated with mVOCs (75 and 100 mM NaCl plants).

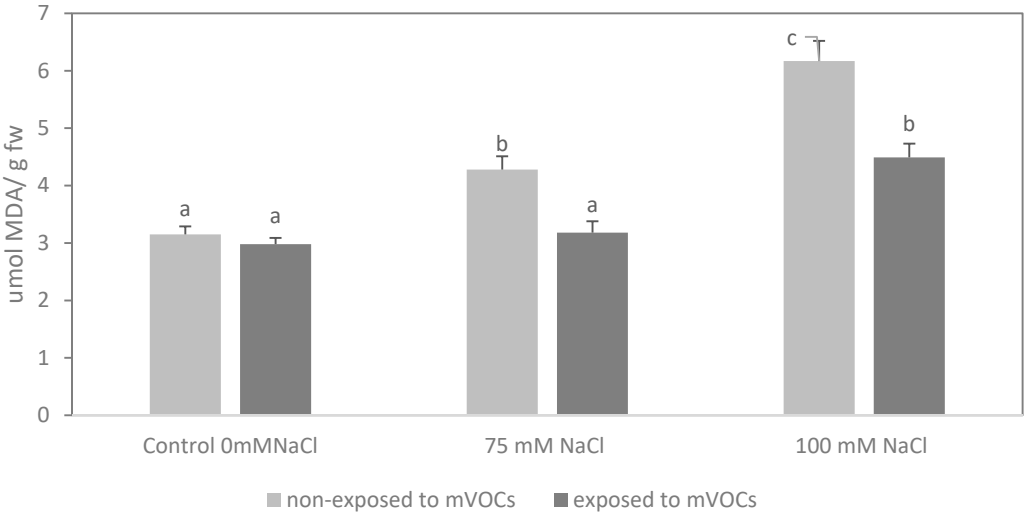

Figure 4. Malondialdehyde (MDA) content in *Mentha piperita* grown under salt stress media (0, 75, and 100 mM NaCl) and exposed to *B. amyloliquefaciens* GB03 mVOCs emission (mean ± SE). Values followed by the same letter in a column are not significantly different according to Fisher's LSD test ($p < 0.05$).

3.5. Principal Component Analysis

PCA represents a graphic image that simplifies the visualization and perception of the dataset and the variables. We used the PCA to extract and reveal the relationships among the factors (growth conditions and exposure to mVOCs) and different variables as EO, TPC, lipid peroxidation (MDA), and antioxidant capacity (AAE) in the multivariate analysis (Figure 5). The plot defined by the first two principal components was enough to explain most of the variations in the data (96.8%) and give

a cophenetic correlation coefficient of 0.997. The PCA (Figure 5) showed that 100 mM NaCl (high salt concentrations) combined with exposure to mVOCs was strongly associated with TPC content and antioxidant capacity (AAE), as revealed by the circle in Figure 5. Considering the relationships among variables, a strong positive correlation (acute angle) was observed between TPC levels and AAE. There were also positive correlations found among MDA levels with no mVOC exposure and 100 mM NaCl. In addition, in PC2, positive relationships were observed between AAE, EO, and TPC with mVOC exposure.

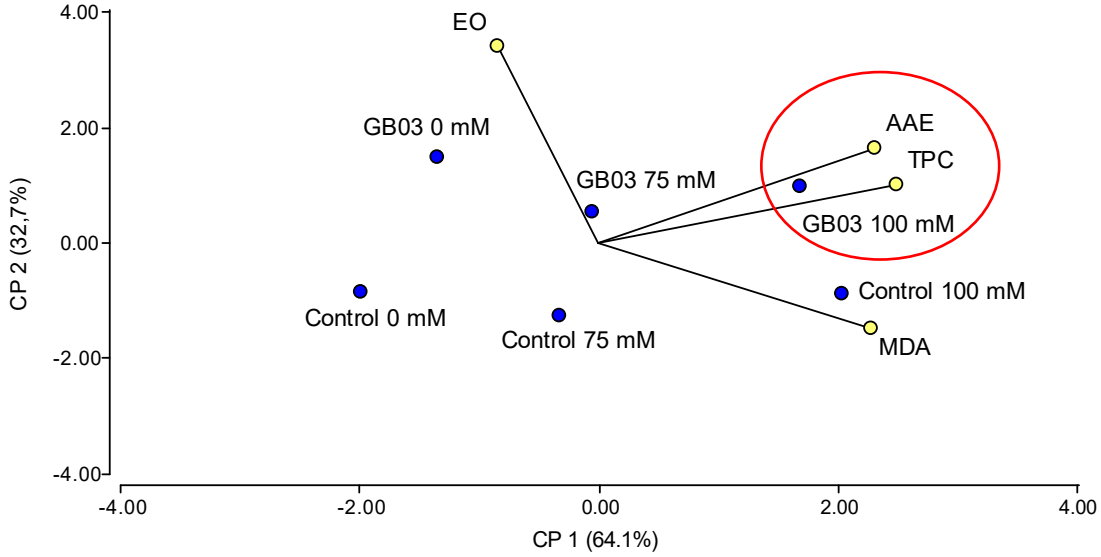

Figure 5. Principal component analysis for the physiological response of *Mentha piperita* grown under different salt stress concentrations (0, 75, and 100 mM NaCl) and *B. amyloliquefaciens* GB03 mVOCs emission. PRO: proline, TPC: total phenolic content, and MDA: lipid peroxidation were determined by estimating the amount of malondialdehyde (MDA); AEE: DPPH radical scavenging capacity.

4. Discussion

Salinity is one of the most important environmental factors diminishing plant yield, mainly in arid and semi-arid environments. The responses of plants to salt stress are intricate and affect several components, with plants having the ability to respond via signal transduction pathways by adjusting their metabolism [45,46]. These responses can differ in relation to toxic ion uptake, ion compartmentation and/or exclusion, osmotic regulation, CO_2 assimilation, photosynthetic electron transport, chlorophyll content and fluorescence, ROS generation, and antioxidant defenses [45–48].

PGPR make a significant contribution to the protection against abiotic stress through their biological activities at the rhizosphere, as exopolysaccharides production (EPS), phytohormones and 1-aminocyclopropane- 1-carboxylate (ACC) deaminase synthesis, induction of the accumulation of osmolytes and antioxidants, upregulating or downregulating the stress responsive genes, and by changes in the root morphology and volatile compounds [17–21,49,50]. In addition, in recent years, an increasing number of PGPR VOC studies have demonstrated an effect against abiotic stresses [7,38,51].

In the present study, we found that when peppermint plants were subjected to salt stress, the EO yield decreased by 50% for both concentrations evaluated (75 and 100 mM NaCl). Additionally, there was a corresponding decrease in the main compounds menthone, menthol, and pulegone. Comparable effects were reported in *M. arvensis* grown under 100, 300, and 500 mM NaCl, with a reduction of 31%, 54%, and 67%, respectively [52]. In contrast, Karray-Bouraoui et al. [53] noted an enhanced *M. pulegium* EO yield of about 2.75-fold under 50-mM salt stress conditions, with a higher density of glandular trichomes on the leaves. Furthermore, Neffati and Marzouk [54] showed that the compounds of *Coriandrum sativum* L. oil were modified by salinity and were revealed to be dependent on salt level treatment. There are contradictory reports concerning changes in EO yield in relation

to salt stress. An increase in EOs and in their composition in response to low levels of salinity was reported in *Satureja hortensis* [55], in sage [56] and in thyme [57]. In contrast, other studies reported a decrease in EOs in lemon balm and in sweet marjoram [58]. Additionally, Ben Taarit et al. [59] reported that the compositions of EOs of *Salvia officinalis* were altered in moderate or high salt stress, in controls and in plants grown under 25 mM NaCl, with the major compound of the EOs being viridiflorol, whereas at higher levels (50 and 75 mM NaCl), 1, 8-cineole was predominant, and at 100 mM NaCl, manool was the principal compound.

The EO yield variations reported under abiotic stress could have resulted from the fact that their production is affected by different physiological, biochemical, metabolic, and genetic factors, which are complex to isolate from one another. In addition, the geographical, seasonal, developmental, and organ variations all contribute to EO yield, as do anatomical and hormonal factors [60–63]. The impact of salt stress on the EO levels probably was due to acclimation processes in stressed plants. Whereas in the initial stage of stress, the metabolism is severely affected, later, the acclimatization processes may reduce the secondary metabolite biosynthesis [64,65].

In the present study, the EO content in salt stressed plants treated with mVOCs showed a 5.6 and 6.5-fold increase with respect to their respective controls (plants grown under 75 or 100 mM NaCl and not treated with mVOCs, respectively), demonstrating that GB03 mVOCs have the capacity to reverse the negative effects of salinity on the EO yield. In fact, mVOCs induced salt tolerance in plants in a previous study of ours, with peppermint plants subjected to salt stress conditions and treated with GB03 VOCs having a higher shoot fresh weight, root dry weight, and total chlorophyll content compared to controls [38]. In this sense, the biosynthesis of terpenoids is affected by the primary metabolism—for example, the photosynthesis for carbon and energy supply. Factors that increase biomass production may have an impact on the relationships among the primary and secondary metabolisms, causing an increased biosynthesis of secondary metabolites [66]. Related to this, augmented plant biomass seems to lead to a larger availability of substrate for monoterpene biosynthesis [35,67].

We have also observed that abscisic acid (ABA) was not connected to salt tolerance generated in plants subjected to salt stress and treated with VOCs [38]. This observation suggests that GB03 VOCs protection against osmosis is ABA independent [68]. The jasmonic acid (JA) levels were similar in salt treated plants, when treated with mVOCs or not. In contrast, the salicylic acid (SA) levels were higher in plants subjected to salt and treated with mVOCs compared to plants subjected to salt conditions and not treated with mVOCs. SA is an important signal molecule for modulating plant responses to stress [38]. Chemical analysis using Solid Phase Microextraction (SPME) fibers of the VOC emissions from GB03 grown under salt conditions revealed the release of a total of seven components, belonging to the following four classes: hydrocarbons (cyclohexane, dodecane, undecane and hexadecane), ketones (acetoin), aldehydes (benzaldehyde), and ethers (2-butanone-3metioxy-3 methyl). The relative quantity of acetoin, the major VOC compound emitted by GB03, enhanced with salt concentration [38]. Concerning the complex profile of compounds, VOC emission is strongly affected by the collection methodology employed, the growth medium, and the density of the bacterium [50,69,70]. For instance, Farag et al. [71] identified a higher number of compounds from GB03 VOCs than Cappellari and Banchio [38], probably due to the different collection methodology used.

It has also been reported that plants treated with GB03 mVOCs and grown in a saline media accumulated less Na + through the regulation of the Na transporter. The GB03 VOCs decreased the Na level in *Arabidopsis* by decreasing Na uptake and/or increasing Na exudation [49]. Furthermore, they led to an acidification of the rhizosphere [72]. Certain bacterial VOCs activate closure of the stomata, reducing the water evaporation [73], and are also involved in biofilm formation, which maintains soil moisture content and increases drought tolerance in plants [51,74,75]. In addition, mVOCs emitted by PGPR also act as a biocontrol against several phytopathogens and trigger plant defense responses through the induction of systemic resistance (ISR) [24,71,76]. For example, the production of EOs is related to the defense response system [63], since numerous terpenes have antimicrobial activity [77]. Similarly, monoterpene synthesis is induced by herbivore feeding in *Minthostachys mollis* [78] and

several plant species, suggesting that these compounds protect leaves from future attacks [67,79–81]. Consequently, as mentioned above, endogenous SA levels increased in plants cultivated under salt conditions and treated with GB03, with previous observations suggesting that the biosynthesis of *M. piperita* monoterpenes is SA and JA dependent [82].

A rise in TPC levels in different tissues under salt conditions has also been described in different plant species [83–85]. A consequence of abiotic stress is superoxide production, which leads to a detoxification mechanism. Related to this, phenolics are synthesized by many plant species for protection against abiotic stress conditions, and their levels are correlated with antioxidant activity [63,86]. Salinity stress induces metabolic and physiological reactions, as well as drastically decreasing the CO_2 uptake due to stomatal restrictions. As a consequence, the consumption of reduction equivalents (NADPH 2+) for CO_2 fixation via the Calvin cycle decreases significantly, leading to oxidative stress and an oversupply of reduction equivalents, with the metabolic processes being moved to biosynthetic activities that consume reduction equivalents. Hence, the biosynthesis of reduced compounds, such as phenols, is increased [63,85,87]. Among the SM found in *M. piperita* are phenolic compounds such as caffeic acid, rosmarinic acid, eriocitrin, and luteolin- 7-O-glucoside [88,89], with their proportion in leaves being approximately 19–23% of dry weight [90–92]. Here, we found that peppermint plants either subjected to salt conditions and/or treated with GB03 VOCs produced a positive effect on the TPC content compared to the respective control plants. Plants grown under 100 mM NaCl and treated with VOCs revealed a higher TPC content. In fact, phenolic compounds are important and powerful agents in scavenging free radicals [93–96]. The antioxidant capacity of phenolic compounds is due to their high reactivity as hydrogen or electron donors, to the particularity of the polyphenol-derived radical to stabilize and delocalize the unpaired electron, and to their capacity to chelate transition metal ions [92,97].

In a previous study, we observed that direct inoculation as well as drought stress in *M. piperita* increased TPC and phenylalanine ammonia lyase (PAL) activity, with the latter being responsible for the synthesis of phenolic compounds [41,43]. In agreement, the TPC was observed to increase in different plant species submitted to abiotic stress [86]—for example, in *T. vulgaris* subjected to drought stress [96] and in *M. pulegium* under salt stress [98]. Conversely, Rahimi et al. [99] and Alhaithloul et al. [100] described a reduction in TPC in *M piperita* plants subjected to drought stress. However, in *Tagetes minuta* plants inoculated with *P. fluorescens* WCS417r and *Azospirillum brasilense,* and in chickpea inoculated with *P. fluorescens* [101], TPC levels increased significantly [36]. Jayapala et al. [102] reported the induction of resistance against pathogens through enhancement of the activities of defense-related enzymes and a higher accumulation of TPC in chili plants inoculated with *Bacillus* sp. Furthermore, Tahir et al. [27] revealed that *Bacillus* sp. mVOCs negatively influence the development of the pathogen *R. solanacearum* by activating ISR in tobacco plants. Molecular studies have shown that resistance is the consequence of an increase in the SM levels and defense-related enzymes, including PAL.

Phenolic compounds are antioxidants that may be required for scavenging ROS and protecting the lipid membrane from oxidative stress [12]. For example, Fagopyrum *esculentum* plants grown under media with increasing salt concentrations revealed a concentration-dependent increase in the accumulation of phenolic compounds, resulting in a higher DPPH free radical scavenging potential [103]. This effect was corroborated in the present study in plants subjected to salinity environments and treated with mVOCs, which showed a heightened antioxidant capacity, as revealed by the high levels of AAE detected in the DPPH• scavenging assay and by the low amounts of MDA. The highest levels of antioxidant activity were observed when plants were grown under 100 mM NaCl and mVOC. The GB03 mVOCs decreased the MDA levels in plants subjected to salt stress, to similar levels as those in control plants. In contrast, after water deficit treatment in peppermint plants, heightened amounts of MDA, as a cell membrane damage index, were detected [99]. Additionally, peppermint growing under control conditions was revealed to be more effective in scavenging DPPH free radicals and had a higher reducing power than when exposed to drought and heat stress. This observation provides

signals that tissues of peppermint subjected to heat and/or drought stress contain fewer antioxidants and reducing compounds [100].

The PCA analysis showed that plants subjected to high salt concentrations combined with exposure to mVOCs strongly affected the TPC content and antioxidant capacity (AAE). This relationship was also detected in drought-stressed peppermint plants inoculated with GB03 [43].

In plants that were inoculated and subjected to osmotic stress, similar results in MDA reduction were observed to those reported for cucumber plants inoculated with a consortium of PGPR under drought stress conditions [104], as well as those in white clover and *M. arvensis* inoculated under saline conditions [51,105]. The decrease in the leaf MDA content resulting from mVOC treatment suggests its ability to reduce the peroxidation of cell membrane lipids under salt stress and to protect the leaf cell from damage. Moreover, Gopinath et al. [106] reported in *Nicotiana tabacum* that when callus was exposed to volatile compounds from *Bacillus badius* M12 and the volatile, 2,3- butanediol, this led to increased antioxidant activity by the expression of SOD, a key antioxidant enzyme. In addition, treatment with mVOCs from GB03 and *Pseudomonas simiae* increased choline and glycine betaine biosynthesis in *Arabidopsis* [51,68]. These osmolytes have positive effects on enzyme and membrane integrity, along with adaptive roles in mediating osmotic adjustment in plants subjected to stress conditions [107]. In another investigation, 2,3-butanediol was found to induce plant production of nitric oxide (NO) and hydrogen peroxide [108], and it was reported that NO regulates antioxidant enzymes at the level of activity and gene expression [109]. At the same time, the plant hormone SA is required for plant growth under abiotic stress [7,17,73]. Finally, an increase in the SA levels was shown in peppermint plants subjected to salt stress and treated with GB03 VOCs [38].

5. Conclusions

Salt stresses affect the growth and productivity of crop plants and are detrimental to the plants, thereby reducing their yield. Thus, it is necessary to improve the technologies of abiotic stress management. In recent decades, several studies have shown that PGPR has the ability to ameliorate the negative effects of salt or water. However, only a few reports have been published on PGPR VOCs as elicitors of tolerance to abiotic stress in aromatic and medicinal plants. The GB03 VOCs have been shown to increase plant growth and chlorophyll content and lead to better morphological characteristics in *M. piperita* plants subjected to salt stress. The results shown in the present study establish that for peppermint plants grown in the laboratory under salt media, the volatiles emitted by GB03 significantly increased SM production and improved the antioxidant status. This suggests that the accumulation of SMs is a plant strategy to avoid oxidative damage caused by ROS, a direct result of salt stress. Bacterial volatiles are promising candidates for a rapid non-invasive technique to increase SM production in aromatic and medicinal crops growing under abiotic stress conditions. In addition, this is a potentially useful system for the production of SMs, which have remarkable biological activities and are often exploited as medicinal and food ingredients for therapeutic, aromatic, and culinary purposes. However, future studies are still necessary to elucidate how plants modulate and perceive PGPR VOC-elicited abiotic tolerance.

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