UNIVERSIDAD DE LA FRONTERA

Facultad de Ingeniería y Ciencias

Programa de Doctorado en Ciencias de Recursos Naturales

EFFECTS OF VOLATILE METABOLITES EMITTED BY SOIL BACTERIA ON GROWTH PROMOTION OF *Lactuca sativa* L.

DOCTORAL THESIS IN FULFILLMENT OF THE REQUERIMENTS FOR THE DEGREE DOCTOR OF SCIENCES IN NATURAL RESOURCES

PAOLA ALEJANDRA FINCHEIRA ROBLES

TEMUCO- CHILE

2017

“EFFECTS OF VOLATILE METABOLITES EMITTED BY SOIL BACTERIA ON GROWTH PROMOTION OF *Lactuca sativa* L.”

This thesis was conducted under the supervision of thesis director Dr. ANDRÉS EDUARDO QUIROZ CORTEZ, in the Departament of Chemical Sciences and Natural Resources at the Universidad de La Frontera and is presented for review by the members of the examining commite.

PAOLA ALEJANDRA FINCHEIRA ROBLES

Director Programa de Doctorado en Ciencias de Recursos Naturales	***Dr. Andres Quiroz***
	Dra. Leonora Mendoza
Director Académico de Postgrado Universidad de La Frontera	***Dr. José Manuel Pérez***
	Dr. Miguel Martínez
	Dra. Ana Mutis
	Dra. Maribel Parada
	Dr. Fernando Borie

… I Thanks the pHD for teaching me so much at this stage ….

Acknowledgement

First, I would like to thank my advisor Dr. Andrés Quiroz. I appreciate his patience, teaching and time dedicated during these years for carrying out this study. Without his guidance and support, this work or my training during the doctorate would not have been possible. I want to thank Dra. Ana Mutis, Dr. Leonardo Parra, Dr. Emilio Hormazabal and members of the examining commitee for the support throughout my doctorate. I give many thanks to my friends, Carolina, Leslie, Lore, Carla and Stephy, for their constant support and advice that helped me during this time. I thank the Laboratorio de Química Ecologica, a nice group of people and my colleagues who taught me the importance of teamwork; I give thanks to Herbert and Manuel for their help and availability when I needed them for carrying out my study. I need to thank Cristian, Felipe, Marcelo and Jocelyn for their important help and companionship during this time.

The most important and my greatest support: my family. My parents and grandparents, I thank them for believing and trusting in me, they all found the words and appropriate times to support me.

I thank all of them for contributing to in my training and make this….. a reality!!!

Summary and outline of this thesis

Nowadays, global feeding is a topic with great relevance given the urgent need to increase food production, so a quick solution is required for it. For compensating this problem, the proposed alternatives are the application of mineral fertilizers (MF) and the use of genetically modified plants (GMP). However, the negative effects of MF on the environment and the lengthy, cumbersome and expensive regulatory normative related to the use of GMP have led to the need to develop new strategies. In this framework, volatile organic compounds (VOCs) emitted by bacterial species emerge as a sustainable alternative to the environmental due to lipophilic property (It prevents leaching to ground water) and fast biodegradability (avoiding the compound accumulation in the environment). Several studies have shown relevant growth-inducing effects of bacterial VOCs at radical (i.e primary root length, lateral root length and root density) and shoot level (i.e fresh weight, chlorophyll content and surface area) of some plant such as *Arabidopsis thaliana* and tobacco. In this sense, *Lactuca sativa* was proposed as a model vegetable due to its easy management, uniform germination and sensibility under environmental conditions. Based on the above, this project thesis proposed to test the following hypothesis: "Volatile organic compounds emitted by soil bacteria isolated from soil promote growth in *L. sativa* seedlings under controlling conditions". To test the hypothesis was proposed to develop the following general objective: "to evaluate the effect of volatile organic compounds emitted by soil bacteria on *L. sativa* grown under controlled conditions". In the first step, the Voges Proskauer test was applied to detect 3-hydroxy-2-butanone (acetoin)-producing soil bacteria as screening tool for selecting potential source of VOCs with growth inducer

effects, allowing 10 selected bacterial species belonging to *Bacillus* (BCT11, BCT9, BCT74-2, BCT5, BCT53, BCT74-1, BCT21 and BCT4), *Serratia* (BCT34) and *Staphylococcus* (BCT54) species (BCT= Derived from the spanish name "Bacteria Cebem Temuco"). Two-day-old seedlings of *L. sativa* were exposed to VOCs emitted by selected bacterial species, which were grown in Murashige & Skoog Agar (MS-A), Nutrient Agar (N-A) and Methyl Red & Voges Proskauer Agar (MRVP-A) for 7 days. The bioassays allowed determining that species belonging to the *Bacillus* genus have a greater capacity to induce growth through VOCs emission compared with Gram negative species. Later, seedlings of *L. sativa* were exposed to VOCs released by BCT4, BCT9 and BCT53 (because of its greater capacity to induce growth) during 10 days, which allowed to select BCT9 strain due to higher ability to elicit growth on *L. sativa*. In the second step, *L. sativa* seedlings were exposed to bacterial VOCs produced by different amount and cell densities of BCT9 grown in MS-A, N-A and MRVP-A. The results indicated that cell densities of $5.0x10^{7}$CFU m^{-1} and $6.0x10^{7}$CFU mL^{-1} induced root length while $2.7x10^{8}$CFU mL^{-1} and $2.0x10^{8}$CFU mL^{-1} elicited shoot length. In addition, lateral root number and dry weight were elicited independent of the applied cellular density. Furthermore, BCT9 grown in MRVP-A showed a rapid growth compared with its cultivation in MS-A, with an intermediate behavior in N-A medium, possibly due to their differential composition. The *Bacillus* sp. BCT9 grown in MRVP-A was selected for the next statement because this combination showed a greater growth-inducing. The respective VOCs emitted by BCT9 grown in MRVP-A were collected and identified through solid phase microextraction (SPME) and gas chromatograph coupled to mass spectrophotometer (GC-MS). The identified compounds were 3-hydroxy-2-butanone, 2,3-butanediol, 2-nonanone, 2-undecanone, 2-tridecanone and 2-pentadecanone. In addition, two delivery systems were

used to evaluate the bioactivity of compounds as inducers of growth: a) controlled (compound mixture with lanolin matrix) and b) abrupt (bioactive compound alone). These VOCs increased root length, dry weight and shoot length at low concentrations (50 and 0.05 ppm), independent from lanolin presence, while lateral root length was elicited only by 2-nonanone (50 ppm) and 2-undecanone (0.05 ppm). Seedlings exposed to 2-nonanone showed the best growth induction at root and shoot level, highlighting its ability to elicit the increase of lateral root length, so it was selected for the next step. Finally, the elucidation of the possible action mechanisms triggered by 2-nonanone to elicit *L. sativa* growth was performed through microscopy technologies (Scanning electron and confocal microscopes). The increase of root hair development was found under abrupt delivery system while an increase in stomatal opening was elicited by controlled delivery system. These results suggest improvement of nutrient and water surface uptake by increase of radical hairs as well as the possible perception and input of VOCs to the plant cell compound by stomata. This thesis provides a relevant evidence to understand the modulation of *L. sativa* growth by VOCs emitted by bacterial species to contribute with a strong knowledge for the new strategy for horticultural management.

TABLE OF CONTENTS

CHAPTER I:

General introduction

1.1 GENERAL INTRODUCTION

Currently, global feeding is an important topic due to both lack of food and nutrition problems, requiring a quick solution because world's human population increased near fourfold in the past 100 years, and it is expected to grow to over 9 billion in 2050 (Nellemann et al., 2009). International policies have focused around this problem because a great number of people still lack food; the latest available estimates indicate that about 795 million people in the world were undernourished during 2014–2016 (Gennari, 2015). Therefore, it is necessary to improve the nutritional quality of food and increase crop quality and quantity (García- Casal, 2007; Newell-McGloughlin, 2008). As already known, vegetable species are one of the main food sources for humans due to their relevant nutritional properties (Pem and Jeewon, 2015).

Vegetables are cultivated under an intensive agriculture, reaching 56,733 ha of surface area at world level with a production of 1,090,425 tones (Gennari, 2015). Vegetable consumption allows maintain a balanced and healthy diet as it contributes with energy, fiber, minerals, vitamins and secondary plant products with health benefits, so its sufficient consumption could help to prevent diseases (Nichols and Hilmi, 2009; Silva dias, 2012). In Chile, vegetable crops are destined to the market of fresh consumption and their export to countries such as Mexico, Brazil, Spain and United Kingdom (Eguillor-Recabarren and Acuña-Reyes, 2016). The cultivation area of vegetables cover 63,775 ha, where corn (9,209 ha), lettuce (6,272 ha) and tomato (4,954 ha) are the main cultivated vegetables.

Chemical products (i.e mineral fertilizer and synthetic phytohormones) are intensely applied in order to achieve maximum efficiency and yield of vegetable production.

Nevertheless, their applications have caused several environmental problems, such as erosion, loss of biological activity and environmental pollution through runoff, leaching and spray components of these products. Besides, toxic substances can be accumulated in vegetables causing adverse effects on organisms that consume them (Savci, 2012). To resolve this problem, the use of economic and natural resources is still considered to develop new technologies to be implemented in vegetables production.

Alternatives strategies derived from Chemical Ecology, specifically semiochemicals that acts as signals compounds are proposed as environmentally sustainable tool to development novel bio-products with the purpose of being implemented in horticulture (Kanchiswamy et al., 2015ab). In the recent years, volatile organic compounds (VOCs) emitted by bacterial species and bioassays have shown strong growth-inducing effects on some plant species through the activation of pathways that regulate physiological processes involved in plant health (Bitas et al., 2013). It is worth mentioning that the growth-inducing effect after exposure to these volatiles can be detected in a period range from 7 days to 30 days (Bailly and Weisskopf, 2012; Kai et al., 2016).

VOCs are characterized by having low molecular weight (< 300 g mol^{-1}), low boiling point, high vapor pressure (0.01 kPa at 20°C) and lipophilic nature, which allow its movement through air, soil and water (Korpi et al., 2009; Kanchiswamy et al., 2015ab). The VOCs may belong to different chemical natures as alkanes, alcohols, ketones, sulfur, among others (Audrain et al., 2015). With the idea of finding bacterial VOCs that trigger vegetables growth, this research has focused in the prospection of soil bacteria as bioactive VOCs sources.

The study performed by Ryu et al. (2003) showed that VOCs released by *Bacillus subtilis* GB03 increased total leaf area of *Arabidopsis thaliana*. Afterwards, Xie et al. (2009) showed that *A. thaliana* exposed to VOCs released by GB03 exhibited an increase in fresh and dry weight. In addition, Gutiérrez-Luna et al. (2010) showed that VOCs emitted by *Bacillus* species increased root development. Later, Orozco-Mosqueda et al. (2013) showed that seedlings of *Medicago truncatula* exposed to *Arthrobacter agilis* UMCV2 increased shoot fresh weight, root fresh weight and chlorophyll concentration. Subsequently, Castulo-Rubio et al. (2015) showed that the exposition to VOCs of *A. agilis* UMCV2 had growth inducer effect on *Sorghum bicolor*. Recently, Asari et al. (2016) reported that seedlings of *A. thaliana* exhibited 2-fold increase in fresh and dry weight after exposition to volatiles emitted by *B. amyloliquefaciens* UCMB5113. Moreover, according to the reported by Velázquez-Becerra et al. (2011), Blom et al. (2011) and Asari et al. (2016), bacterial culture conditions (i.e culture medium and inoculum concentrations) strongly determine the VOCs bacterial profile to induce growth.

The gas chromatography coupled with mass spectrometry has allowed to identify growth inducers belonging to different chemical natures such as 2,3-butanediol, 2-pentylfuran, dimethylhexadecylamine, β-caryophyllene, dimethyl disulfide and acetophenone, which act at low concentrations in a range from microgram to nanogram (Ryu et al., 2003; Zou et al., 2010; Velázquez-Becerra et al., 2011; Minerdi et al., 2011; Meldau et al., 2013; Groenhagen et al., 2013; Ann et al., 2013; Bhattacharyya et al., 2015; Ditengou et al., 2015).

In order to understant about the action mechanisms related to VOCs effects on plants, some studies have elucidated the pathways that are modulated during exposure to VOCs.

The ability of VOCs to induce plant growth is attributed to changes at physiological level, which involve principally the modulation of essential nutrients, hormonal balance and sugar concentrations (Zhang et al., 2007, 2009; Bailly et al., 2014). Some reports have shown that VOCs released by *B. subtilis* GB03 elicited the increase of iron availability (Zhang et al., 2009; Orozco-Mosqueda et al., 2013; Castulo-Rubio et al., 2015). It should be mentioned that Fe availability is closely related to photosynthesis process, which can be modulated by VOCs released by *B. subtilis* GB03 through hexokinases pathways dependence and abscisic acid concentration modulation. In addition, differentiated genetic profile expression has been determined after exposure to bacterial VOCs associated with to metabolism (i.e lipid and flavonoid), growth (i.e expansin and pectinases), stress (i.e antioxidant activity), cellular signaling (i.e secondary messengers) and phytohormones signaling pathways (i.e auxin, brassinosteroid, gibberalins, cytokinin and ethylene) (Zhang et al., 2007; Minerdi et al., 2011; Kim et al., 2015; Hao et al., 2016). These findings have shown the key role that bacterial VOCs have as molecular signals to elicit plant growth through the activation of physiological pathways.

According to the statements described above, *Lactuca sativa* L (lettuce) is proposed as a vegetable model to prospect VOCs effects on horticulture with the purpose of implementing these signal compounds as new technology and strategy to reduce adverse effects produced by traditional chemical products. *L. sativa* was chosen due to its wide use as substrate in toxicity studies because of its easy management, rapid germination and growth, high sensibility, and uniform germination under various environmental conditions (Valerio et al., 2007; Bagur-González et al., 2010).

The observations derived from reported study cases constitute a strong evidence supporting that bacterial VOCs can be applied to horticulture crops as a friendly and new alternative. Therefore, a novel field of research for the elucidation of signal molecules that trigger growth promotion on vegetable species and subsequent design for sustainable strategies has arisen due to the little information available so far. It is noteworthy that chemical nature of VOCs determines their rapid biodegradability and low probability of leaching. Therefore, they are proposed for the development of approaches related to VOCs application.

Considering that bacterial VOCs can be a new and friendly tool, emerge the need to develop an effective selecting method for detecting bacterial species with ability to produce VOCs that elicit growth induction. In addition, the identification of bioactive VOCs and its optimal doses represent the opportunity to scale greenhouse-level application approach. Therefore, main focus of this thesis was to identify VOCs released from bacterial species responsible for inducing the growth on *L. sativa* seedlings and elucidates its possible mechanism.

1.2 HYPOTHESIS

Under this framework, reports about volatile organic compounds (VOCs) emitted by bacterial species have provided relevant evidence about their growth inducer activity at root and shoot levels. Therefore, VOCs emerge as interesting molecules to be implemented in vegetable species, taking into account their ability to act as signal molecule to activate physiological effects associated with growth promoting activity. From these approaches the

following thesis hypothesis arises.

- Volatile organic compounds emitted by bacteria isolated from soil promote growth in *Lactuca sativa* L. seedlings under controlled conditions.

1.3 GENERAL OBJECTIVE

To evaluate the growth induction elicited by volatile organic compounds emitted by soil bacteria on *Lactuca sativa* grown under controlled conditions.

1.4 SPECIFIC OBJECTIVES

1.4.1 To select soil bacteria with growth promoting activity on *Lactuca sativa* seedlings by volatile organic compound emission.

1.4.2 To optimize bacterial culture conditions for the emission of volatile organic compounds for promoting growth on *Lactuca sativa* seedlings.

1.4.3 To determine volatile organic compounds emitted by soil bacteria involved in promoting growth on *Lactuca sativa* seedlings.

1.4.4 To elucidate possible action mechanisms elicited by the more active volatile organic compound for inducing *Lactuca sativa* seedling growth.

CHAPTER II:

Microbial volatiles as plant growth inducers

2.1 INTRODUCTION

Currently, the high demand for food and the needs for increasing both performance and quality of agricultural crops have led to the applications of large amounts of chemical products (i.e mineral fertilizer and commercial phytohormones), which have been used primarily to increase nutrient availability and stimulate the growth of species grown under field and greenhouse conditions, respectively (Zaman et al., 2015). Nevertheless, their applications have caused serious environmental problem, resulting in loss of soil biological activity and erosion derived from runoff, leaching and spray components of these products (Savci, 2012). In addition, the synthetic compounds applied in greenhouse conditions have caused food contamination associated to toxic substance accumulation (i.e nitrosamine compounds in lettuce) (Ward, 2009). Therefore, the search for sustainable alternatives has been performed in order to reduce the input of chemical products in crops and producing chemical-free foods, emerging the microorganisms associated with rhizosphere as potential growth inducers.

Microorganisms both bacteria and fungi are found in high quantity and diversity in the rhizosphere zone, defined as “the narrow zone surrounding influenced by plant roots and characterized by their intense association with microbial activity” (Mendes et al., 2013; Dessaux et al., 2016; Van Dam and Bouwmeester, 2016). These microorganisms utilize root exudates, which contain primary and secondary plant metabolites, i.e. organic acids, phytosiderophores, sugars, vitamins and amino acids, representing from 20 to 40% of fixed carbon located in underground root system (Philippot et al., 2013; Venturi and Keel, 2016). The plant exudates can determine or modify microbial community along the root system (Badri et al., 2009). Otherwise, microorganisms secrete diverse non volatile metabolites

with beneficial effects to induce plant growth through direct and indirect pathways (Dotaniya and Meena, 2014). Several studies performed in the last decades indicate that direct pathways involve the released phytohormones (i.e auxin, ethylene and cytokinins) and organic substances (i.e organic acids) that contributed in growth stimulation and nutrient availability, respectively. Indirect pathways comprise substances that prevent pathogens attack through hydrolytic enzymes, antibiotics, siderophores and hydrogen cyanide (Goswami et al., 2016; Vejan et al., 2016). However, a new mechanism mediated by volatile organic compounds (VOCs) was reported for the first time by Ryu et al. (2003), who showed that volatiles released by *Bacillus subtilis* GB03 induce growth on *Arabidopsis thaliana*, being the first evidence that volatile organic compounds can modulate growth, stress, nutrition and health processes in plant.

Studies performed under controlled conditions using bi-comparment Petri plates, which have a central partition that only allows the interaction between microbial strain tested and seedlings target through the head-space, have revealed that compounds as 2,3-butanediol, 3-hydroxy-2-butanone and 2-pentylfuran can induce growth at radical or foliar level in *A. thaliana* and tobacco (Ryu et al., 2003; Zou et al., 2010; Ann et al., 2013). Furthermore, studies have shown that some microbial species belonging to the *Bacillus*, *Burkholderia*, *Arthrobacter*, *Laccaria*, *Pseudomonas*, *Fusarium*, *Alternaria* or *Trichoderma* have the ability to emit VOCs that induce plant growth. While, plant species used for testing volatiles have been *Mentha piperita*, *Medicago truncatula*, *Medicago sativa*, *Nicotiana tabacum*, *Zea mays* or *Glycine max* (Gutiérrez-Luna et al., 2010; Blom et al., 2011; Velázquez- Becerra et al., 2011; Minerdi et al.2011; Ditengou et al., 2015; Park et al., 2015; Sánchez-Lopez et al., 2016).

In summary, microbial volatiles represent a novel alternative as sustainable bio-product with potential use in agricultural crops for reducing chemical products (Piechulla and Degenhardt, 2014; Kanchiswamy et al., 2015a). Until now, studies have highlighted that growth induction at radical and foliar level depends on the following points: (1) specific interaction seedling-microbial species, (2) culture conditions and (3) the concentration of bioactive compound. Furthermore, action mechanisms at cellular, physiological and molecular level have been scarcely elucidated. This review has focused on study cases, covering seedlings target, microbial tested and inducer effects as well as the identified compound as growth elicitors with special focus on action mechanisms activated by VOCs.

2. 2 Microbial VOCs: Concept and chemical properties

Microbial VOCs are signal molecules with low molecular weight (< 300 g mol^{-1}), low boiling point, high vapor pressure (0.01 kPa at 20°C) and lipophilic nature that acts as ideal info chemical for modulating physiological processes, traveling through the air, soil and water (Kanchiswamy et al., 2015b). The VOCs released from a determinate microorganism has a specific profile that includes compounds derived from different microbial metabolic pathways depending on living environment; some compounds belongs to alkanes, alkenes, alcohols, esters, ketones, terpenoids and sulfur families (Schulz and Dickschat, 2007; Korpi et al., 2009; Audrain et al., 2015). The VOCs are produced by microorganisms in a given range of scales and play a key role as signaling molecule that can act as a wide range of stimuli giving rise to the activation of a series of signals, which regulate physiological processes involved in plant health (Bailly and Weisskopf, 2012;

Bitas et al., 2013; Kai et al., 2016). In the next statement, study cases that provide relevant evidence showing the role of VOCs as growth inducers are described.

2.3. Plant growth elicited by microbial VOCs: Case studies

Several studies about the inducer effects of bioactive VOCs on plant seedlings have been carried out since 2003 to date (Table 1). The first study was performed by Ryu et al. (2003), who showed that volatiles released by *B. subtilis* GB03 elicited a ~5.6-fold increase of total leaf area of *A. thaliana* after 10 days of exposition. Later, Banchio et al. (2009) showed that the same bacterial species increased growth on shoot-root biomass of *Ocimum basilicum*, which increased 2-fold respect to control while leaf surface was increased ~ 2.5-fold. Furthermore, Xie et al. (2009) showed that *A. thaliana* seedlings exposed to volatiles released by GB03 exhibited 58 and 71% increases in fresh and dry weight after 2 weeks of exposition. The same interaction was tested by Zhang et al. (2009), who showed that chlorophyll concentration in *A. thaliana* had 84% increases. Subsequently, Kwon et al. (2010) showed that GB03 elicited significantly the increase of root and shoot fresh weight on *A. thaliana*, after 6 days of inoculation.

Additionally, others *Bacillus* strains have been tested as growth inducers through the emission of volatiles. Zou et al. (2010) showed that volatiles emitted by *B. megaterium* XTBG-34 exhibited a 1.7-fold increase in fresh weight of *A. thaliana* on day 7. Additionally, the effect of VOCs on root system was demonstrated by Gutiérrez-Luna et al. (2010), showing that volatile emitted by *Bacillus* species modified root architecture, eliciting the increase of total fresh weight, primary root length, lateral root number and

lateral root length on *A. thaliana* as well as evidenced a strong association between fresh weight and lateral root length on day 10. Afterwards, Santoro et al. (2011) showed that volatiles emitted by *B. subtilis* caused the increase of root dry weight (3.5-fold) and shoot fresh weight (2-fold) on *Mentha piperita*. Later, Meldau et al. (2013) reported that *Nicottiana atenuata* exposed to volatiles released from *Bacillus* sp. B55 exhibited 5-fold increase in leaf surface and true leaves were enhanced in ~233%. In addition, the exposition to B55 increased lateral root for cm^{-1} over 400% compared with control. Furthermore, Ann et al. (2013) indicated that volatiles emitted by *B. vallismortis* EXTN-1 induced the increase of fresh weight in tobacco with high percentage. Recently, Hao et al. (2016) reported that volatiles released from *B. amyloliquefaciens* FZB42 induced the increase of dry and fresh weight on *A. thaliana* and a study performed by Asari et al. (2016) reported that seedlings of *A. thaliana* exhibited 2-fold increase in fresh and dry weight after exposition to volatiles emitted from *B. amyloliquefaciens*.

Other bacterial species belong to Gram positive species have been reported for its ability to release volatile organic compounds with growth inducer activity. A study performed by Velázquez-Becerra et al. (2011) reported that *Arthrobacter agilis* UMCV2 had the ability to emit VOCs inducing growth in *Medicago sativa*, enhancing plant fresh weight (~ 40 mg versus ~ 60 mg), stem length (~ 3.0 cm respect to ~1.7 cm) and lateral root density (~2.5 versus ~1.7). Later, Orozco-Mosqueda et al (2013) showed that seedlings of *Medicago truncatula* exposed to volatiles released from *A. agilis* UMCV2 for 5 days increased shoot fresh weight, root fresh weight and chlorophyll concentrations in 40%, 35% and 35%, respectively. Later, a study performed by Castulo-Rubio et al. (2015) showed that the exposition to VOCs of *A. agilis* UMCV2 had growth inducer effect on *Sorghum*

bicolor, increasing shoot fresh weight in 66%. Besides, Lee et al. (2012) reported that *Paenibacillus polymyxa* E681 emitted a volatile mixture that elicited the increase of surface leaf area foliar (1.6-fold) and fresh weight enhances 2-fold.

On other hand, Gram negative species have been shown to emit volatile compounds with growth-promoting activity. A study performed by Blom et al. (2011) reported that bacterial species belonging to *Burkholderia* and *Chromobacterium* genera increased biomass on *A. thaliana* between ~ 200 and 300%. Subsequently, Groenhagen et al. (2013) reported that exposition of *A. thaliana* to volatiles released from *Burkholderia ambifaria* LMG19182 increased the number of lateral root number around 222% as well as the shoot biomass in 260%. Furthermore, Bailly et al. (2014) indicated that *A. thaliana* exhibited 3-fold increase in plant biomass and number of lateral root after exposition to volatiles released from *Escherichia coli*. Furthermore, Bhattacharyya et al. (2015) showed that *A. thaliana* exposed during 14 days to volatiles from *Proteus vulgaris* JBLS202 exhibited a 75-80% increase in fresh weight and induced an increase in primary root length and shoot length by 33.3-37.1% and 24.4-26.7% respectively. In addition, Park et al. (2015) reported that tobacco seedlings had 8.8 and 9.5-fold increase in fresh weight and dry weight respectively, after exposition to volatiles released from *Pseudomonas fluorecens* SS101 and Vaishnav et al. (2015) reported that *Glycine max*. L Merril exposed to volatiles from *Pseudomonas simiae* strain AU exhibited a 58, 86 and 58% of increase in shoot length, root length and fresh weight respectively.

Additionally, some fungi species have been reported for emitting bioactive compounds to induce plant growth. Minerdi et al. (2011) reported that volatiles released from *Fusarium oxysporum* MSA35 induced the growth of root length (95.6%), shoot length (75%), fresh

weight (85.8%), chlorophyll content (68%) and the number of lateral root (3-fold). Later, Paul and Park (2013) indicated that tobacco fresh weight was increased in 567% after 4 weeks exposed to VOCs released by *Cladosporum cladospoiodes* CL-1. Besides, Hung et al. (2013) showed that *A. thaliana* exhibited 45% and 58% increase in total biomass and chlorophyll concentration, respectively. In addition, Ditengou et al. (2015) reported that *A. thaliana* seedlings exposed to volatiles released from *Laccaria bicolor* exhibited 2-fold increase in number of lateral roots. Subsequently, Bitas et al. (2015) studied the effects of volatile compounds on 46 *Fusarium oxysporum* strains, but only the isolates NRRL 26379 and NRRL 38335 induced increase in shoot weight, leaf surface area, chlorophyll content, root mass and root length by 2.5-3.0, 2.7-4.0, 3, 4.8-4.4, 3.6-5.2 fold respectively. Recently, Sánchez-López et al. (2016) showed that volatiles released from *Alternaria alternata* induced the increase of plant height on *A. thaliana* and *Zea mays* with a greater percentage (nearly 2-fold). The studies presented above indicated that growth inducer activity mediated is elicited by diverse microbial species including fungal and bacterial species. According to the description in Table 1, 55% studies have focused on *A. thaliana*, as model plant whereas 45% of case studies include other species as *S. bicolor*, *M. sativa*, *M. piperita*, *O. basilicum*, *L. sativa*, *Z. mays*, *C. annuum*, *M. truncatula*, *N. attenuata* and *G. max*. The principal factors that determine the emission of a specific microbial VOC profile under controlled and field conditions are described in the next statement.

Table 1. Case studies of growth induction via volatiles organic compounds on different plant-microorganisms interactions.

Microorganism	Genus/ Strain	Plant	Culture medium	Exposition	Growth parameter	Reference
Bacteria	*B. subtilis* GB03	*A. thaliana*	MS-A	10 days	Surface leaf area	Ryu et al. (2003)
	B. subtilis GB03	*A. thaliana*	TSA	3 weeks	Fresh weight Dry weight	Xie et al.(2009)
	B. subtilis GB03	*O. basilicum*	MS-A	14 days	Leaf area Shoot fresh weight Root fresh weight	Banchio et al. (2009)
	B. subtilis GB03	*A. thaliana*	MS-A	14 days	Chlorophyll content	Zhang et al. (2009)
	B. megaterium XTBG-34	*A. thaliana*	TSA	7 days	Fresh weight	Zou et al. (2010)
	Bacillus strains	*A. thaliana*	MS-A	10 days	Total fresh weight Primary root length Lateral root number Lateral root length	Gutiérrez-Luna et al. (2010)
	B. pyrrocinia Bcc171 *C. violaceum* CV0	*A. thaliana*	Angle-A MRVP-A LB-A MS-A	21 days	Plant fresh weight	Blom et al. (2011)
	P. fluorecens *B. subtilis* *A. brasilense*	*M. piperita*	MS-A	30 days	Shoot fresh weight Root dry weight	Santoro et al. (2011)
	P. polymyxa	*A. thaliana*	MS-A	2 weeks	Leaf surface area	Lee et al. (2012)

B. subtilis GB03				Foliar fresh weight	
B. ambifaria	*A. thaliana*	LB-A	3 weeks	Lateral root number Shoot biomass	Groenhagen et al. (2013)
Bacillus sp. B55	*N. attenuata*	YPDA	12 days	Leaf surface True leaf Lateral root cm^{-1} Root length	Meldau et al. (2013)
B. vallismortis EXT-1	Tobacco	TSA PDA KBA LB-A N-A WA	7 days	Fresh weight	Ann et al. (2013)
A. agilis UMCV2	*M. truncatula*	N-A	5 days	Shoot length Root length Shoot fresh weight Root fresh weight Stem chlorophyll	Orozco-Mosqueda et al. (2013)
E. coli	*A. thaliana*	MS-A	14 and 21 days	Biomass Secondary roots	Bailly et al. (2014)
A. Agilis UMCV2	*S. bicolor*	N-A	2 days	Shoot fresh weight Root fresh weight	Castulo-Rubio et al. (2015)
A. Agilis UMCV2	*M. sativa*	N-A	6 days	Plant fresh weight Stem length Lateral root density	Velázquez-Becerra et al. (2015)

	P. vulgaris	*A. thaliana*	LB-A	14 days	Fresh weight Root length Shoot length Number of lateral root	Bhattacharyya et al. (2015)
	P. fluorescens SS101	Tobacco	King B	3 weeks	Fresh weght Dry weight	Park et al. (2015)
	P. simiae AU	*G. max*	King B	10 days	Shoot length Root length Fresh weight Number of lateral root Leaf surface area	Vaishnav et al.(2015)
	B. amyloliquefaciens strains	*A. thaliana*	TSA LB-A M9A	18 days	Dry weight Fresh weight	Asari et al. (2016)
	B. amyloliquefaciens FZB42	*A. thaliana*	MS-A	16 and 23 days	Fresh weight Dry weight	Hao et al. (2016)
Fungi	*F. oxysporum* and bacterial consortium	*L. sativa*	CMA	7 and 14 days	Root length Seedling fresh weight Shoot length Leaf chlorophyll content	Minerdi et al. (2011)
	C. cladosporioides	Tobacco	PDA	4 weeks	Fresh weight	Paul and Park (2013)
	Trichoderma	*A.thaliana*	MEA	4 weeks	Total biomass chlorophyll concentration	Hung et al. (2013)
	L. bicolor	*A.thaliana*	PM P20 A	10 days	Lateral root development	Ditengou et al. (2015)

F. oxysporum strains	*A. thaliana* Tobacco	PDA	14 days	Shoot fresh weight Total leaf area Chlorophyll content Root length Root fresh weight Lateral root density	Bitas et al. (2015)
A. alternata	*A. thaliana* *Z. mays* *C. annuum*	M9A	12-50 days	Plant height Total carotenoids Photosynthetic parameters	Sánchez-López et al. (2016)

Abbreviations: MS-A: Murashige and Skoog medium agar, Angle-A: Angle agar, MRVP-A: Methyl Red Voges Proskauer agar, LB-A: Luria Bertani agar, N-A: Nutrient agar, PM20 -A: Pachlewski medium P20 A, TSA: Tryptic Soy agar, PDA: Potato Dextrose agar, KBA: King's B agar, WA: Water agar, YPDA: Yeast Peptone Dextrose agar. MEA: Malt Extract agar. CMA: Complete Medium agar. M9A: Minimal Medium agar.

2.4 Conditions involved in the emission of microbial VOCs

The emission of specific VOCs profile depends strongly on the environment in which the specific microorganism grows. The experiments performed under controlled conditions have shown that a single bacterial strain may induce or inhibit the growth depending on medium in which it grows (Asari et al., 2016; Blom et al., 2011; Velázquez-Becerra et al., 2011). Some culture media used for microorganisms grown have been MRVP-A, MS-A and N-A (Bailly and Weisskopf, 2012). MRVP-A medium has been used for enhancing the production of 3-hydroxy-2-butanone and 2,3-butanediol. The MS-A has been utilized in several previous reports as a medium for bacteria growth, and N-A has been used in studies involving *M. sativa* growth (Ryu et al., 2003; Velázquez-Becerra et al., 2011). The culture media are composed differentially, MRVP-A containing glucose as carbon source and pH 6.9 ± 0.2. Besides, N-A is composed by beef extract and peptone with pH 6.8 ± 0.2; while MS-A contains mineral nutrients with sucrose as C source and lower (pH 5.7). Therefore, the different culture medium composition can affects directly the production of volatile organic compounds released by metabolic pathways of microorganisms, so their bioactivity can depends strongly on these factors (Blom et al., 2011). Additionally, a study performed by Fincheira et al. (2016a) showed that some bacterial genus can have a stronger effect compared with other genera i.e. *Bacillus* species emitted volatile compounds with greater effects to induce growth on *L. sativa* seedlings compared with Gram negative species as *Pseudomonas*, *Staphylococcus* and *Serratia* species independent of culture media used (MRVP-A, MS-A and N-A).

Other parameters that determine the modulator effect on seedlings is the amount or concentration of applied inoculums. Velázquez-Becerra et al. (2011) reported a dose-

dependence response of *M. sativa* exposed to VOCs release by *A. agilis* UMCV2, reaching the best increase on root length, root density, stem length and fresh weight with 50 µL of inoculum grown in N-A was applied compared with doses from 100 to 500 µL. Afterwards, Blom et al. (2011) showed that *Burkholderia pyrrocinia* Bcc171 increased dry weight on *A. thaliana* when grown in LB-A and MRVP-A, reaching the best yield with 10 µl of applied inoculums. Recently, Asari *et al*. (2016) showed that VOCs released by *B. amyloliquefaciens* UCMB5113 induced a significant increase on dry weight of *A. thaliana* (phyllosphere) when quantities from 20 to 100 µL of inoculum were applied on LB-A, minimal medium (M9) or Tryptic Soy agar.

Under field conditions, VOCs profile emitted by microorganisms depends on soil properties, microbial community, plant exudates and internal factors that influence the metabolism of each microbial strain (Kai et al., 2016). Soil physicochemical properties as pH, oxygen, T°, water, inorganic particle size, mineral aggregates and size and shape of pores determine a microclimate for microbial growth influencing their lifecycle. Additionally, the relation of specific strain with microbial community through intra and inter specific relation can modulate the production and distribution of volatiles, altering the profile in response to external stimuli (Kai et al., 2016). In respect to root exudates, they play a nutritional role for microorganisms present in the rhizosphere (biochemical cycles), whereby plant species, age and environmental conditions produce different rhizodeposition influencing soil microbial diversity (Bulgarelli et al., 2013). Other important factors are microbial growth rate, the state of development of metabolism, the biofilm formation and spore generation of a specific strain that can modify the emission and concentration of VOCs (Chen et al., 2015). The VOCs can be adsorbed, desorpted or reacted with clay

surfaces as well as diffuse through soil, water or air in the rhizosphere (Ramirez et al., 2009; Insam and Seewald, 2010) (Figure 1). The bioactive compounds with proven growth inducing activity are described in the next statement.

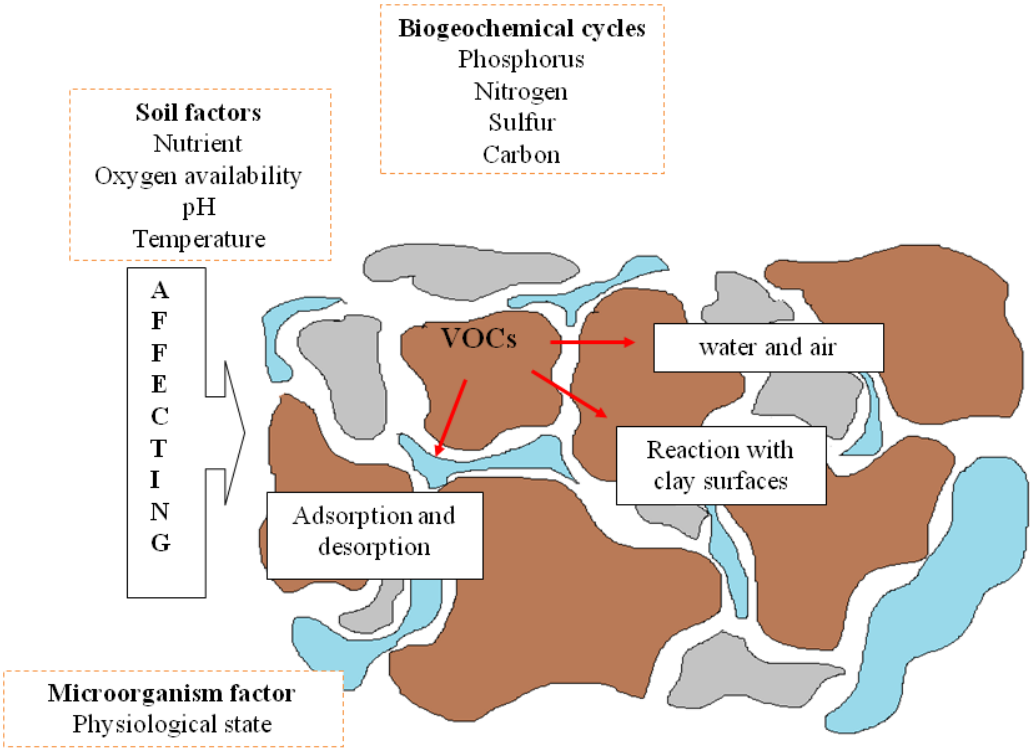


Figure 1. Soil factors that contributed to emit a specific microbial VOCs profile (Brown= soil, grey= mineral particles, blue= water).

2.5 Identified bioactive microbial volatiles as growth inducers

In the last years, diverse chemical compounds emitted by metabolism of bacteria and fungi have been identified by gas chromatography coupled to mass spectrometry, reaching more than 200 compounds according to Korpi et al. (2009). These compounds are produced from primary (i.e derived from aminoacids and fatty acids) and secondary (derived of side

products from primary metabolism) metabolisms (Schulz and Dickschat, 2007). The bioactive VOCs identified as growth inducers belong to different chemical natures as alcohols, ketones, sulfur compounds, furans and terpenes that act at low concentrations (Figure 2, Table 2). The first identified compound was reported by Ryu et al. (2003), who showed that 2,3-butanediol induced increase of the surface leaf area in *A. thaliana* when was applied between 1 and 100 μg. Later, Zou et al. (2010) indicated that 2-pentylfuran elicited the increase of fresh weight in the same plant species at 0.5 μg μL^{-1}. Whereas, Velázquez-Becerra et al. (2011) reported that dimethylhexadecylamine (8- 32 μM) induced the increase of fresh weight, stem length, root length and root density on *M. sativa.* β-caryophyllene at doses from 25 to 100 μM induced the enhancement of root length, shoot length, fresh weight and chlorophyll on *L. sativa* seedlings (Minerdi et al., 2011). Afterwards, Meldau et al. (2013) reported that dimethyl disulfide can act as sulfur source contributing to nutrition on tobacco seedlings with an optime dose of 50 μM. Whereas, Groenhagen et al. (2013) reported that dimethyl disulfide and acetophenone elicited the increase of biomass in *A. thaliana* at doses of 1 ng μL^{-1} and 1 μg μL^{-1}, and Ann et al. (2013) showed that 3-hydroxy-2-butanone acts as an elicitor of increasing fresh weight at 1 and 10 ppm on tobacco. Subsequently, Bailly et al. (2014) and Bhattacharyya et al. (2015) reported that indole at low doses induced growth on *A. thaliana.* More recently, studies performed in 2015 showed new compounds as growth inducers. A study performed by Park et al. (2015) indicated that 13-tetradecadien-1-ol, 2-methyl-η-1-tridecene and 2-butanone at 5 and 50 ng induced fresh weight on tobacco. Ditengou et al. (2015) reported that (-)-thujopsene induced lateral root formation at 100 ppb on *A. thaliana*. Table 2 shows the different solvents used to apply bioactive volatiles on bioassays, highlighting the use of distilled

water (i.e 3-hydroxy-2-butanone, dimethyhexadecylamine and indole) and dichlorometane (i.e 2,3-butanediol, acetophenone and 1-hexanol).

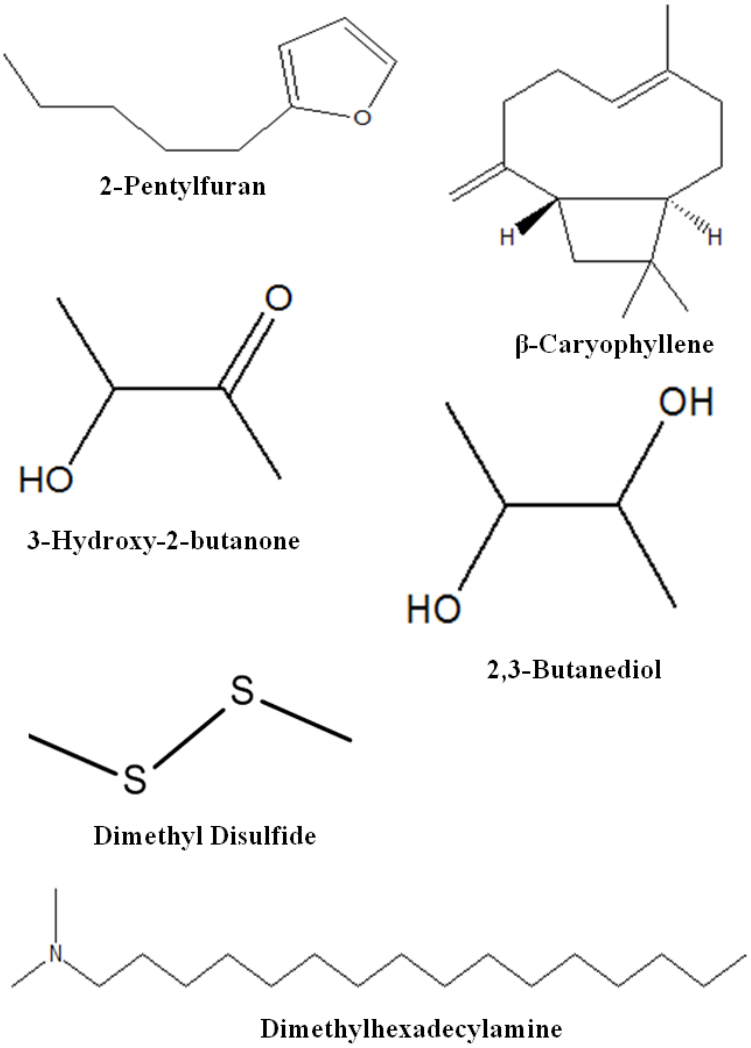


Figure 2. Chemical structure of microbial VOC involved in promoting plant growth.

Table 2. Bioactive microbial volatiles as growth inducers.

Compound	Solvent	Dose range tested	Optime dose	Seedling target	Parameter	Exposition	Reference
2,3-Butanediol	Dichlorometane	1000 mg 10 mg 100 µg 1 µg 0.01 µg	100 µg 1 µg	*A. thaliana*	Surface leaf area	14 days	Ryu et al. (2003)
2-Pentylfuran	Dichloromethane or Alcohol	1 mg 20 μL^{-1} 100 µg 20 μL^{-1} 10 µg 20 μL^{-1} 1 µg 20 μL^{-1} 0.1 µg 20 μL^{-1}	10 µg 20 μL^{-1}	*A. thaliana*	Fresh weight	15 days	Zou et al. (2010)
Indole 1-Hexanol Pentadecano	Dichlorometane	1 ng 10 μL^{-1} 10 ng 10 μL^{-1} 100 ng 10 μL^{-1} 10 µg 10 μL^{-1} 1 mg 10 μL^{-1}	10 µg 10 μL^{-1} 1 mg 10 μL^{-1}	*A. thaliana*	Fresh weight	21 days	Blom et al.(2011)
β-Caryophyllene	Distilled water	25 µM 50 µM 100 µM	25 µM 50 µM 100 µM	*L. sativa*	Root length Shoot length Fresh weight Chlorophyll	7 days	Minerdi et al. (2011)
Dimethylhexadecylamine	Distilled water	4 µM 8 µM	8 µM 32 µM	*M. sativa*	Fresh weight Stem length	10 days	Velázquez-Becerra et al. (2011)

		16 μM 32 μM 64 μM			Root length Lateral root density		
Dimethyl disulfide	Methanol	50 μM 1000 μM	50 μM	*A. thaliana*	Lateral root number	17 days	Meldau et al. (2013)
3-Hydroxy-2-butanone	Distilled water	0.001 ppm 0.01 ppm 0.1 ppm 1 ppm 10 ppm	1 ppm 10 ppm	Tobacco	Fresh weight	1 week	Ann et al. (2013)
Dimethyl disulfide Acetophenone	Dichlorometane	1 ng 1 μg 1 mg	1 μg μL^{-1} 1 ng μL^{-1} 1 ng μL^{-1}	*A. thaliana*	biomass	3 weeks	Groenhagen et al. (2013)
Indole	Distillled water	10 nM 100 μM	10 nM	*A. thaliana*	Biomass Secondary roots	14 and 21 days	Bailly et al.(2014)
Indole	Dichlorometane	0.001 μg μL^{-1} 0.005 μg μL^{-1} 0.01 μg μL^{-1} 0.02 μg μL^{-1} 0.043 μg μL^{-1} 0.080 μg μL^{-1} 0.120 μg μL^{-1} 0.250 μg μL^{-1} 0.500 μg μL^{-1}	0.01 μg μL^{-1} 0.02 μg μL^{-1} 0.043 μg μL^{-1} 0.080 μg μL^{-1} 0.120 μg μL^{-1} 0.250 μg μL^{-1}	*A. thaliana*	Shoot length Primary root length Lateral root number Fresh weight	14 days	Bhattacharyya et al. (2015)

		1 μg μL^{-1} 10 μg μL^{-1}					
13-Tetradecadien-1-ol 2-Methyl-η-1-tridecene 2-Butanone	Metanol	5 ng 50 ng 500 ng	50 ng 5 ng	Tobacco	Fresh weight	4 weeks	Park et al. (2015)
(-)-Thujopsene	n-Pentadecane	1 p.p.b 10 p.p.b 100 p.p.b 1000 p.p.b	100 p.p.b	*A. thaliana*	Lateral root formation	10 days	Ditengou et al.(2015)

In the next point, we discuss the action mechanisms associated with growth inducer effects of volatiles emitted by microorganisms with a specific plant "target".

2. 6 Action mechanisms associated with VOCs effects.

In the last years, some studies have reported physiological and molecular effects on plant seedlings in response to microbial volatile exposition in plant-microbe interactions. The studies have shown that VOCs can induce growth principally by three mechanisms that modulate essential nutrients, hormonal balance and sugar concentrations. It highlights that changes related to genes associated with cellular structures and metabolisms are heavily regulated according to Zhang et al. (2007).

Iron is an intensively studied essential micronutrient due to its importance in photosynthesis process, participating in reactions associated with electron transference and photosynthetic machine (Kim and Guerinot, 2007; Waldvogel-Abramowski et al., 2015). Two strategies are used by plants to acquire iron from soil. The strategy I consists of proton exudation, reduction Fe^{+3} to Fe^{+2} and the importation of Fe^{+2} as well as their association with *FIT1* (Fe-deficiency-induced-transcription), *FRO2* (Ferric reductase) and *IRT1* (Iron – regulated transporter 1), where *IRT1* and *FRO2* are regulated by *FIT1*, which codified a protein that regulates the response of plant to iron deficiency. Furthermore, strategy II is associated with phytosiderophores, where Fe can be directly transported into root without its reduction due to the presence of specific transporters in plants (Waldvogel-Abramowski et al., 2015). In relation to that, Zhang et al. (2009) reported that *B. subtilis* GB03 volatiles induced direct and indirect acidification of rhizosphere of *A. thaliana*. Furthermore, GB03 activated transcriptionally Fe uptake, where the expression of *IRT1* was UP-regulated 10-20 fold 2-4 days post exposition. The transcript abundance of *FRO2* increases within 2 days; activating the acquisition of Fe by the strategy I. Based on the above mentioned both strategies are activated to increase Fe content after three days of volatile exposition (30%).

Parallel to the induction of expression of *FRO2* and *IRT1* the seedlings exhibited an increase in the accumulation of *FIT1* transcript after exposition to GB03. Therefore, GB03 increased photosynthesis through Fe assimilation, which is supported by the increase of photosynthetic capacity (F_V/F_m) and chlorophyll content. Later, Orozco-Mosqueda et al. (2013) showed relevant evidence about Fe acquisition on *M. truncatula* after exposition to volatiles released from *A. agilis* UMCV2, which induced acidification of *M. truncatula* rhizosphere after 24-48 h Fe stress. Furthermore, seedlings exposed to dimethylhexadecylamine (DMHA) exhibited a similar acidification after 48 h. Ferric chelate reductase activity at root level was increase up to 120% after exposition to VOCs released by *A. agilis* under Fe deficiency (after 24 hr of stress). Moreover, VOCs of UMCV2 induced the increased of chlorophyll content (35%) and foliar (40%) and root (30%) growth. Recently, Castulo-Rubio et al. (2015) reported that seedlings of *S. bicolor* exposed to VOCs of UMCV2 induced the increase of chlorophyll concentration after deficiency and sufficiency of Fe with VOCs and DMHA; while lateral root number and shoot fresh weight was increased under Fe sufficiency. The study at molecular level indicated that relative transcription level of *FRO1* increased after exposition to UMCV2 volatiles under sufficiency and deficiency of Fe.

A phenomenon strongly associated with the nutritional status of iron is a photosynthesis process that involves the conversion of light energy into chemical energy through the sugar production. High sugar level induces storage processes and gives feedback inhibition of photosynthesis, were hexokinases play a relevant role acting as glucose sensors. Zhang et al. (2008) reported that volatiles released by GB03 increased photosynthetic activity and chlorophyll content (88%), observing greener plants due to the

increase of chloroplast units and the induction of photosynthetic genes as chlorophyll a/b binding protein (CAB2) and Rubisco subunit binding protein. Therefore, photosynthetic activity of photosystem II (PSII) and the maximum and effective quantum yields of PSII were increased. In contrast, quantum yield of non-photochemical dissipation in PSII was reduced. Besides, GB03 VOCs suppress plant sugar sensing as indicated chlorophyll accumulation and the coexistence of increased endogenous photosynthesis and sugar (hexokinase dependent pathway). Signal transduction sugar dependent hexokinase requires Abscicic acid (ABA) signaling and GB03 reduces its levels through the reduction of expression of genes related to ABA-synthesis and response genes to ABA. Recently, Sanchez-López et al. (2016) reported that VOCs released by *A. alternata* increased photosynthetic parameters in leaves, enhancing total carotenoids and chlorophyll, so net rate of CO_2 assimilation and rate of electron transport as well as VOCs increased soluble sugars (sucrose, glucose and fructose). Furthermore, this study indicated that VOCs elicited growth through cytokinin pathway, increasing its active forms. However, the induction of increase in fresh weight and starch was carried out only under diurnal conditions.

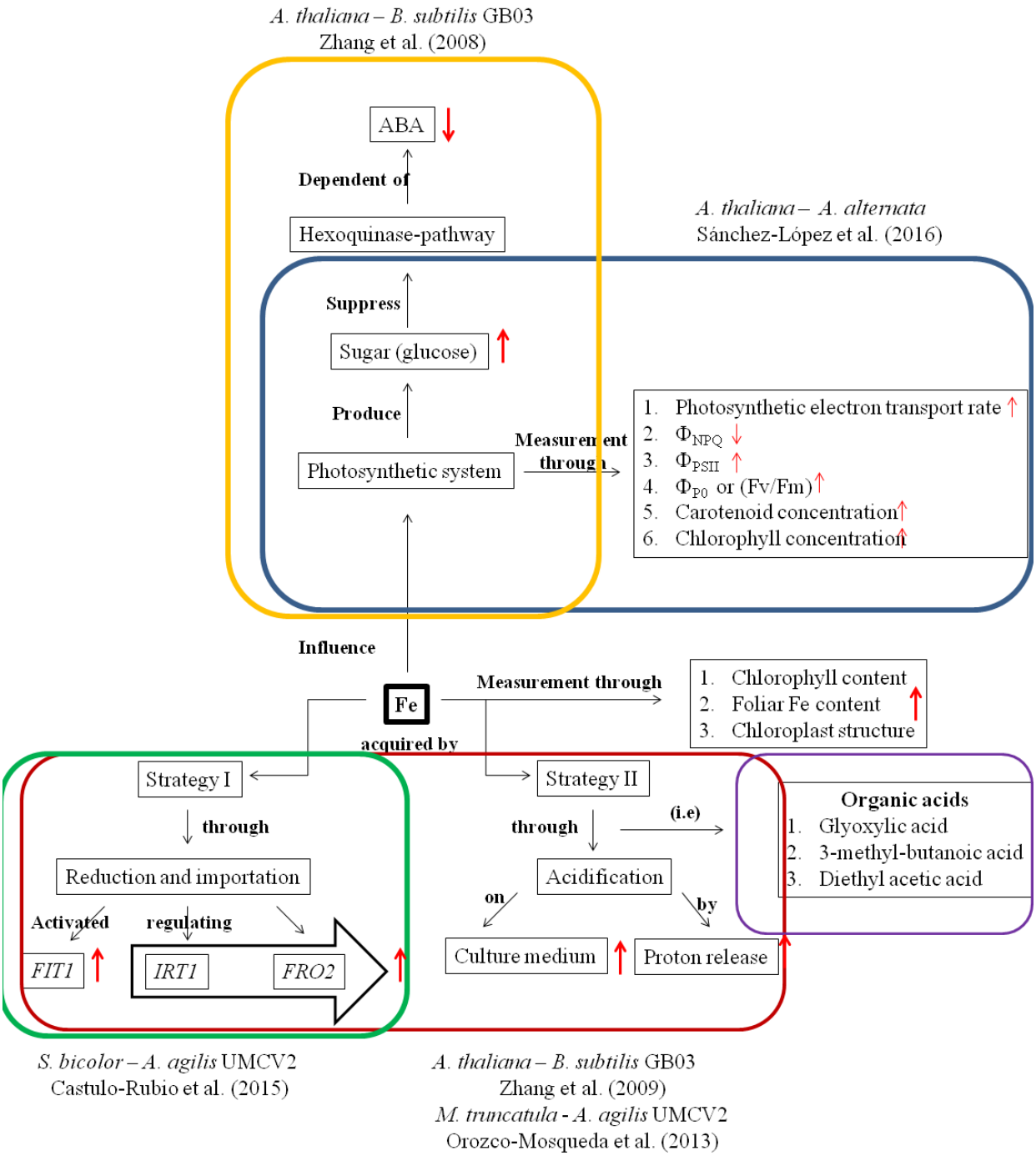


Figure 3. The effects of VOCs emitted by microbial species on Fe acquisition and their cell implications. Different *grey lines* styles indicate the information contributed by different authors. *Abbreviation*: Abscisic acid (ABA); *Genes: FIT1* (Fe-deficiency-induced-transcription), *FRO2* (Ferric reductase), and *IRT1* (Iron–regulated transporter 1). *Symbology:* F_V/F_m= Photosynthetic capacity, ϕ_{PSII}= Effective quantitative efficiency of Photosystem II, ϕ_{NPQ}= Efficiency of heat dissipation.Color lines indicated the information contributed by different authors (↑=Increase, ↓= Decrease) (Figure was performed by authors).

Some bacterial strains have shown important effects to modulate genetic expression in seedlings exposed to VOCs. The first evidence was reported by Zhang et al. (2007), who performed a transcriptomic analysis in *A. thaliana* seedlings exposed to GB03 VOCs during 48 and 72 h, revealing differential expression of genes associated with metabolism, growth, stress and cellular signaling. It highlights that genes associated with auxin, including synthesis and responsive genes were up-regulated, whereas genes associated with auxin transport were down-regulated. Furthermore, genes associated with cell wall modification were regulated by GB03 VOCs, covering up regulation of expansins, which promote cell wall expansion as well as down-regulated pectate lyases and pectinases, reducing cell wall rigidity. Genes as *EXP5*, *NIT1* and *NIT2* were strongly up-regulated after 72 h exposition at foliar level. In addition, Minerdi et al. (2011) showed that VOCs released by *F. oxysporum* and its bacterial consortium induced expansin A5 gene expression in lettuce seedlings.

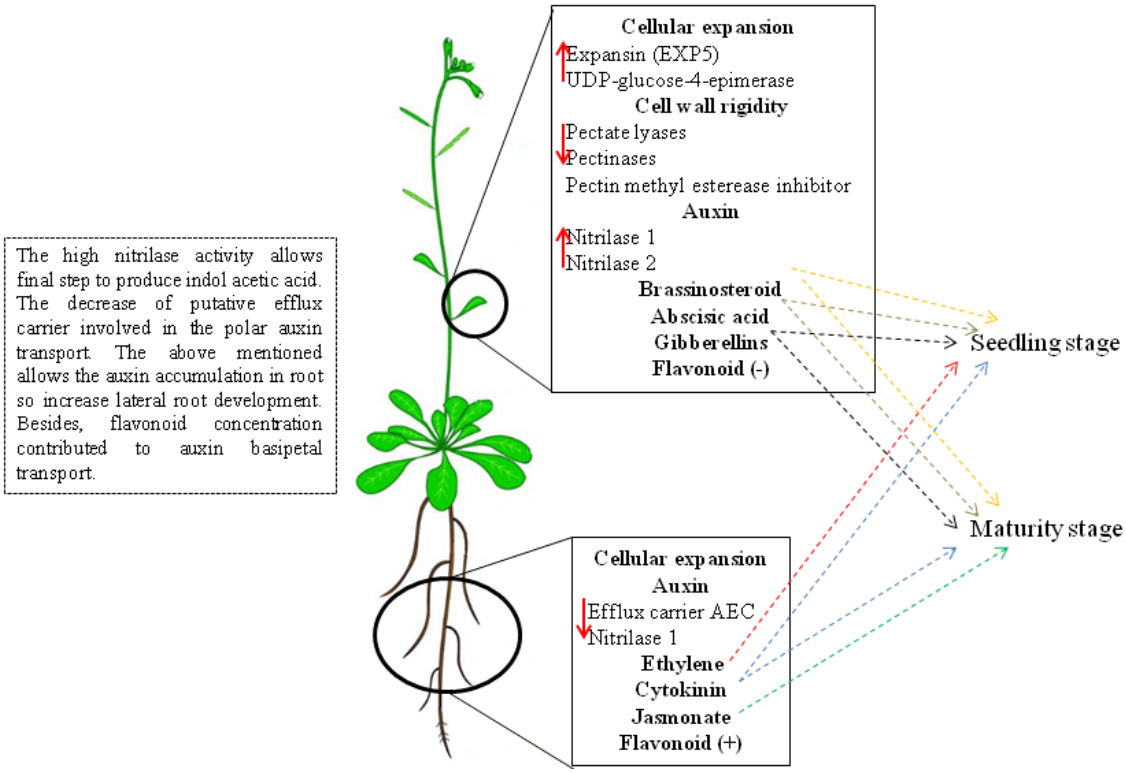


Figure 4. Microbial VOCs effects on cell expansion and phytohormone modulation at root and foliar level during seedling and maturity stage (↑ = increase, ↓ = decrease). (Figure was performed by author).

Later, Kim et al. (2015) showed that volatiles released by *B. subtilis* strain JS had the ability to modulate gene profile expression in tobacco seedlings during metabolic and cellular processes. The up-regulated genes were chlorophyll a/b binding protein, cellulose synthase, acyl-ACP-thioesterase, succinyl-coA ligase alpha I unit, chloroplast sedoheptulose-1,7-biphosphate, sucrose transporter, MLO-like protein 1,cytosolic NADP-malic enzyme and P-protein of glycine decarboxylase; while down-regulated genes were glucosyltransferase, nitrate reductase, methionine-R-sulfoxide reductase B4 protein, glutathione S-transferase and carboxylase. Recently, Hao et al. (2016) indicated that *A.*

thaliana exposed to volatiles emitted by *B. amyloliquefaciens* FZB42 induced differential expression in genes associated with plant hormones, cell wall modifications and protection against stress situations depending on specific (root and leaves) tissue and growth stage (seedlings and mature).

Additionally, some studies have investigated the phytohormones signaling pathways implicated in growth promotion induced by VOCs. A study performed by Ryu et al. (2003) showed that *B. subtilis* GB03 increased surface leaf area through cytokinin.signaling pathway. Later, Bailly et al. (2014) reported that indole had a relevant role to modulate secondary root development in *A. thaliana* through auxin signaling. The bioassays showed that indole acts on zones of auxin activity and during auxin polar transport to induce response. The results indicated that indole accumulation produced alterations in root physiology for increasing lateral root formation. Additionally, this study points out that seedlings respond mostly to indole respect to synthetic auxin, suggesting that bioactive compound induces early development of lateral roots controlling the auxin physiology. Subsequently, Bhattacharyya et al (2015) reported that *P. vulgaris* JBLS202 stimulated growth on *A. thaliana* through auxin, cytokinin and brassinosteroid pathways, which was demonstrated through *Arabidopsis* mutants with disruptions in hormone production and signaling of auxin (*eir1*), cytokinin (*cre1*) and brassinosteroid (*cbb1*). Furthermore, bioassays performed at genetic level corroborated the results with seedling mutants, where *SAUR* (auxin response-gene), *AHK1* (induced in response to citokinin), *CPDA* (associated with biosynthetic pathway of brassinosteroid) and *ERF* (representative of ethylene) were up regulated; while *GA3OX3* (catalyzes conversion of gibberellins precursor in their bioactive compounds) was down-regulated. Additionally, the presence of enzyme inhibitors as

aminoethoxyvinylglycine and propiconazole supported the results mentioned above. In the same year, Bitas et al. (2015) reported that volatiles released by *F. oxysporum* induced growth on *A. thaliana* through auxin signaling and transport. At the same time, Ditengou et al. (2015) reported changes of sesquiterpenes profile at radical level in *A. thaliana* eliciting the increase on root hair length through ROS-dependent mechanism, associated with the generation of superoxide anion radicals (O_2^-) in roots, independently from auxin signaling.

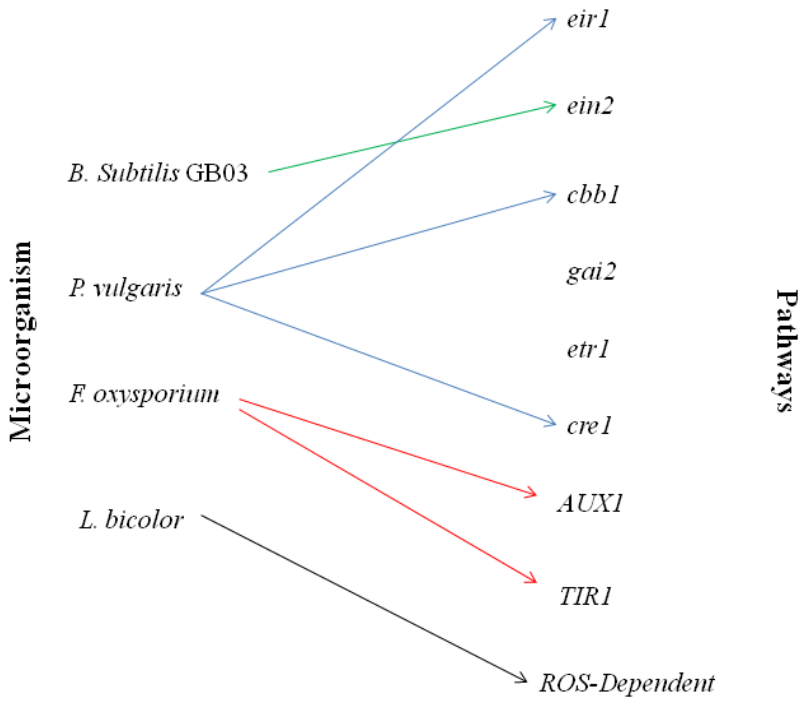


Figure 5. Pathways involved eliciting growth on *A. thaliana* after exposition to volatiles released by different microbial VOCs. Each color line associates the microorganism and the pathway by which it induces growth. (*eir1*= auxin transport deficient and ethylene-insensitive, *ein2*= ethylene-insensitive, *cbb1*= insensitive to brassinosteroid, *gai2*= insensitive to gibberellic acid, *etr1*= ethylene-insensitive, *cre1*= ethylene-insensitive, *AUX1*= genes encode a repressor of auxin response, *TIR1*= gene encodes an F-box protein, a component of the ubiquitin ligase complex that degrades the AUX/IAA transcriptional repressor proteins). (Figure was performed by author).

The study performed at proteomic level by Kwon et al. (2010) showed that GB03 volatiles modulated proteomic expression related to cellular location, molecular function

and biological processes, highlighting that proteins associated with response to stimulus, suggests that the seedlings recognize bacterial volatile as exogenous stimuli, it is emphasized that catalase, glutathione reductase, superoxidase dismutase and dehydroascorbate reductase activities increase after exposition to GB03 volatiles. Up to date, we described different case studies that involve VOCs as growth elicitor; identified compounds and action mechanisms that attempt to explain the action mode of these compounds. In the next statement are appointed. The principal perspectives respect to VOCs application in agriculture and horticulture.

2. 7 Perspectives and conclusions

Recent advances have showed that VOCs emitted by microorganisms associated with root plants can be a novel strategy to apply as growth inducers with potential use in agricultural species. From the initial studies that have contributed with relevant evidence that VOCs have the ability to act as signal molecule for eliciting growth, the need to research the emission of volatiles from diverse microorganisms and its ability to act on one or more plant species emerge. The second challenge is the evaluation of the specificity of single or mixture compounds previously identified under laboratory conditions to check their capacity to induce growth, characterizing their specific composition and action mode. To date, research on action mode of a specific compound to determine its effect on the regulation of cellular and metabolic processes to elicit growth should be elucidated, so, proteomic, molecular and metabolomic techniques must be carried out to achieve a better understanding. In addition, greater progress is required to implement the application of VOCs under field conditions. Thus, experimental setups should be designed in order to

investigate and standardize methodologies and formulations to mimic rhizosphere conditions. Novel techniques will help to evaluate effects on plant growth required in order to minimize discrepancies showing that microbial VOCs can be a novel technology to be applied in agricultural crops, as well as, the search of novel alternatives to provide sustainable agricultural products that farmers and consumers need. Therefore, VOCs emerge as novel product and strategy that could provide a sustainable alternative with potential use in agriculture.

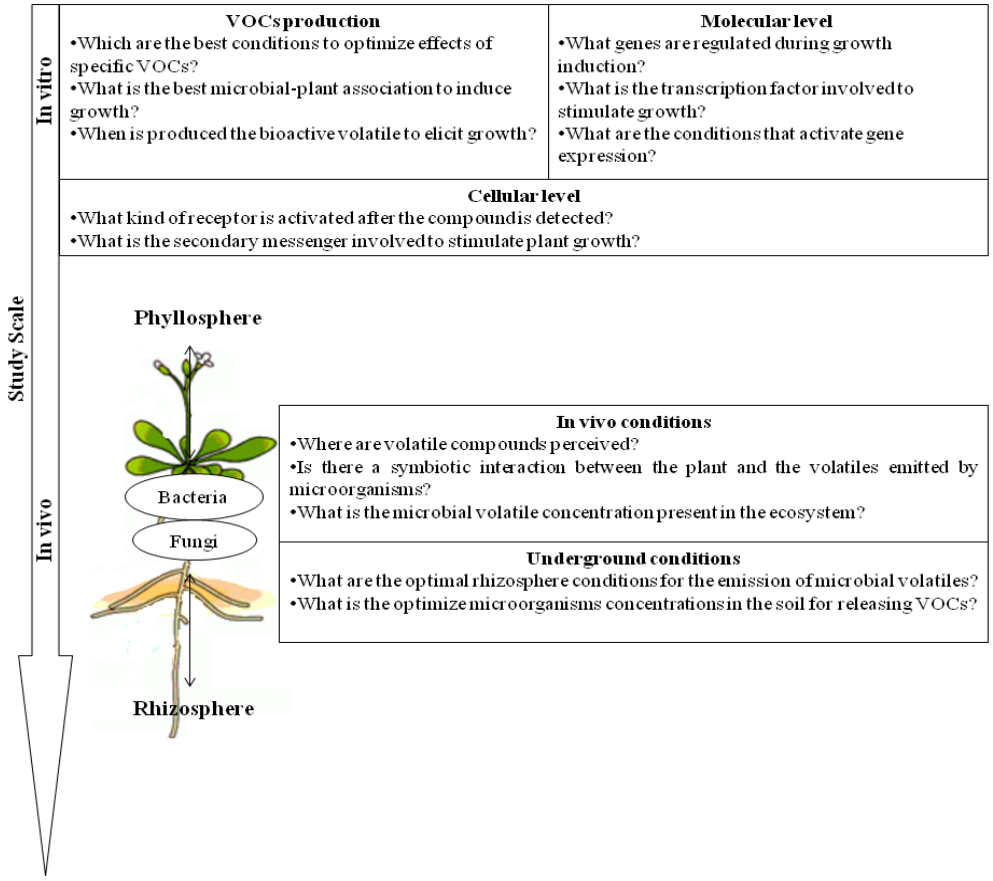


Figure 6. Perspectives for future directions to research microbial VOCs as growth inducers under controlled and field conditions.

CHAPTER III:

Growth promotion of *Lactuca sativa* in response to volatile organic compounds emitted from diverse bacterial species

3.1 INTRODUCTION

Currently, diverse chemical products are applied in horticulture to increase crop production. Nevertheless, new environmentally sustainable alternatives to reduce pesticides, fertilizers and synthetic phytohormones are being investigated (Kanchiswamy et al., 2015ab). Based on this trend, bacteria have emerged as an alternative option to use in horticultural crop production through the formation of non-volatile metabolites, which are related to the production of phytohormones, nitrogen fixation, phosphate solubilization, siderophore production and antibiotics (Bhattacharyya and Jha, 2012). However, in the last decade, a new mechanism that increases plant growth has been reported; volatile organic compounds (VOCs) released from bacteria establishing an aerial contact with the target plant (Bitas et al., 2013). These compounds are described as lipophilic compounds with low polarity and low molecular weight (<300 g mol^{-1}), derived from natural biosynthetic pathways of microorganisms. Furthermore, these VOCs have a high vapor pressure (0.01 kPa) under environmental conditions, allowing their vaporization and easy release (Ortíz-Castro et al., 2009). These compounds can act as ideal signal molecules for mediating interactions at both short and long distances (Kanchiswamy et al., 2015a). Some bacterial species belonging to the genera *Bacillus*, *Burkholderia, Serratia*, *Pseudomonas* and *Arthrobacter* have been studied mainly for their ability to produce these VOCs as growth inducers. The first study performed by Ryu et al. (2003) documented that VOCs release by *Bacillus subtilis* GB03 increased total leaf surface area in *Arabidopsis thaliana*. Later, Zou et al. (2010) reported that *B. megaterium* strain XTBG34 increased the fresh weight of the same species. Likewise, Gutiérrez-Luna et al. (2010) observed that some *Bacillus* sp., which was isolated from soil associated with *Citrus aurantifolia,* emitted VOCs, inducing an increase in *A. thaliana* primary root length, lateral root number and lateral root length.

Moreover, Blom et al. (2011) documented that volatiles released from *Burkholderia pyrrocinia* Bcc171 elicited an increase in *A. thaliana* shoot fresh weight. Likewise, Groenhagen et al. (2013) indicated that different *B. ambifaria* strains increased biomass in *A. thaliana*. In addition, *Arthrobacter agilis* UMCV2 stimulated the growth of shoot and roots of *Medicago sativa* and *M. truncatula* (Orozco-Mosqueda et al., 2013; Velázquez-Becerra et al., 2011). Furthermore, VOCs emitted by *Pseudomonas fluorecens* elicited leaf area growth in *Mentha piperita* (Santoro et al., 2011). More recently, Park et al. (2015) indicated that *P. fluorescens* strain SS101 promoted growth in tobacco through the increase in fresh weight. There is little information available related to growth effect of VOCs in *Serratia* and *Staphylococcus* genera. Kai and Piechulla (2009) reported that volatiles emitted by *Serratia odorifera* 4Rx13 increased relative fresh weight of *A. thaliana* and Verpermann et al. (2007) observed that *Staphylococcus epidermidis* increased growth in the same species.

Considering the diversity of soil bacteria releasing VOCs with growth inducing activity at foliar and root level, the necessity to develop a quick and effective screening tool of bacterial isolates (Gram+/Gram-) emerges as key factor for selecting a potential source of VOCs growth inducers. The conventional biochemical test known as "Voges Proskauer" allows for the detection of bacterial species with ability to produce 3-hydroxy-2-butanone (3H2B or acetoin). This compound can be emitted by Gram +/- species belonging to several genera, such as *Bacillus, Micrococcus, Klebsiella*, and *Acinetobacter* (Xiao and Xu, 2007). Acetoin has shown to be a potential growth inducer in tobacco and can induce resistance in *A. thaliana* (Rudrappa et al., 2010; Ann et al., 2013).

The main model plant used as target of VOCs with growth inducing ability has been *A. thaliana* (Bailly and Weisskopf, 2012). However, there are scarce studies related to the application of growth inducing VOCs on other cultivated plants, such as horticultural species. In this study, *Lactuca sativa* was used as a model horticulture plant to investigate prospective bacterial VOCs as growth inducers. *L. sativa* is characterized by fast germination and growth, easy management and sensitivity to environmental conditions. Hence, this species is widely used to evaluate phytotoxicity effects of both pure compounds and complex mixtures (Torres, 2003; Charles et al., 2011; Díaz de Villegas et al., 2011). Therefore, the objectives of the present study were: (1) to evaluate a Voges Proskauer test as a rapid bacterial screening tool for detecting VOCs as growth inducers, (2) to evaluate different bacterial strains and culture media for releasing VOCs with growth-inducing activity and (3) to determine the effects of 3H2B on *L. sativa* growth.

3.2 MATERIAL AND METHODS

3.2.1 Sample collection. A soil sample was collected in La Araucanía Region (Chile) (38° 58'21" south latitude and 72° 7' 21.7" west longitude) belonging to the Andisol soil series.

3.2.2 Isolation of bacterial strains. Ten grams of the soil sample were suspended in 90 mL sterile distilled water and stirred (120 rpm) for 5 min. One milliliter of this solution was used to perform a serial dilution in a 1:10 ratio (sample solution: sterile distilled water). Then, 100 µL of each dilution were deposited onto Plate Count Agar (PCA) plates (Difco® Laboratories, Detroit, MI) and incubated at 28°C in darkness to obtain isolated colonies. Finally, bacterial isolates obtained were kept at -20°C in peptone agar containing 15% glycerol.

3.2.3 Selection of bacterial isolate producing 3-hydroxy-2-butanone (3H2B). The obtained bacterial isolates were subjected to the Voges Proskauer (VP) test for detecting the bacterial isolates with the ability to produce 3H2B, according to the method described by Romick and Fleming (1998), including some modifications. To perform a qualitative detection, 0.6 mL reactive A (5% alpha-naphthol dissolved in absolute ethyl alcohol)and 0.2 mL reactive B (40% KOH dissolved in sterile distilled water) were added into 4 mL of a bacterial suspension grown in methyl Red Voges Proskauer broth (MRVP-B) (Merck®) at 28°C and centrifuged at 160 rpm. In addition, *Pseudomonas* sp. BCT42 was included as the control, as it does not produce of 3H2B.

3.2.4 Genetic characterization of bacterial isolates (16S rRNA analysis). Based on the screening of bacterial isolates producing 3H2B, 11 selected strains were characterized according to 16S rRNA gene sequencing. Total DNA was extracted using a microbial DNA isolation kit (Ultraclean®, Mo-Bio Laboratories). The 16srRNA gene was amplified by polymerase chain reaction (PCR) with bacterial universal primer set: 27f (5`- AGA GTT TGA TCC TGG CTC AG- 3`) and 1492r (5`- TAC GGY TAC CTT GTT ACG ACT T-3`). PCR was performed in a total volume of 25 µL containing Pfx® amplification buffer, 1 µL of 10 pmol/ul 27f primer, 1 µL of 10 pmol/ul 1492r primer, 2 µL of template DNA, 0.5 µL of $MgSO_4$ (50 Mm), 0.75 µL of dNTP mix (10 mM), 0.2 µL of pfx® amplification enzyme and 14.5 µL of water. PCR conditions consisted of a hot-start at 94°C for 5 min, followed by 35 cycles of denaturation at 94°C for 1 min, annealing at 52°C for 1 min, and extension at 72°C for 2 min. Amplified fragments were developed through agarose gel electrophoresis and purified using FavorPrep™ ®Gel/PCR purification kit. The 16s fragments were sequenced by Macrogen Corporation (Seoul, Korea). The obtained DNA sequences were compared with existing

sequences in GenBank by using the BLAST tool (Campos et al., 2014; Peace et al., 1994).

3.2.5 Phylogenetic analysis. To determine phylogenetic relationships and evolutionary distances among the bacteria, a multiple sequence alignment was conducted with all the DNA sequences identified in this study by ClustalW. From the aligned sequences, a maximum likelihood tree was constructed using MEGA6.06 (Tamura et al., 2013). A Kimura 2 parameter model plus Gamma distribution (G) was selected as the best DNA model determined by the software. The complete deletion option was selected to eliminate all the positions containing gaps and missing data. To evaluate the reliability of the tree, 1000 bootstrap replications were performed according to Jones et al. (1992).

3.2.6 Biochemical assays. Biochemical characterizations of bacterial isolates were conducted by different tests, including indol (IND), methyl red (MR), voges proskauer (VP), catalase (CAT), starch hydrolysis (SH), protein hydrolysis (PH) and gelatin liquefaction (GL) (Parvathi et al., 2009). The response of each strain to different salinities (7% and 14% NaCl), temperatures (10°C, 18°C, 28°C, 35°C and 45°C) and pH (pH 4, pH 7 and pH 11) were determined based on strain growth through the application of 5 µL of each bacterial species suspended (10^7 CFU mL^{-1}) in physiological solution (NaCl 0.85% w/v) onto PCA, adjusted to the conditions mentioned previously (modified from Zahid et al., 2015).

3.2.7 Carbohydrate fermentation analysis. The evaluation of carbohydrate metabolism was performed using an API 50 CH system (BioMerieux®, France). After overnight incubation, single colonies from bacterial isolates were transferred to a physiological solution (NaCl 0.85% w/v) to obtain a bacterial solution with concentration of 2 on the McFarland scale through commercial API-standard (BioMerieux®, France). Then, the

bacterial solution was added to API 50 CHB/E medium (BioMerieux®, France) to inoculate test strips and incubate for 24-48 hours at 28°C. The method for determining carbohydrate fermentation was conducted according to instructions provided by the kit manufacturer.

3.2.8 Plant material and growth conditions. Commercial seeds of *L. sativa* (Green lettuce cv Reina de Mayo asepo, semillas Fito, S.A, Barcelona, Spain) were sterilized with sodium hypochlorite for 8 min and washed with sterile distilled water. Then, the seeds were placed on Petri dishes with Murashige and Skoog Basal medium supplemented with vitamins 0.5X (PhytoTechnology® Laboratories, LLC™) containing 0.8% agar and 1.5% sucrose. Petri dishes were stored in an incubator at temperature maintained between 20 and 25°C with a 16:8-h light: dark cycle and 36-W fluorescent lights. Finally, seedlings were removed after 2 days for bioassays (Minerdi et al., 2011; Ryu et al., 2003).

3.2.9 *Lactuca sativa* growth promotion by VOCs. One day prior to the start of the growth promotion experiments, the selected bacterial isolates were transferred into Falcon tubes with 3 mL nutrient broth (N-B) (Difco® Laboratories, Detroit, MI) and cultured overnight. Subsequently, aliquots of the bacterial suspensions were inoculated into new Falcon tubes with 4 mL N-B to obtain an absorbance (AB) of 0.1 at 600 nm (10^7 CFU mL^{-1}) for the VOC bioassays. Experiments were performed in two compartment Petri dishes (100 x 15 mm) (Greiner Bio-One, Chile), with two 2 day-old *L. sativa* seedlings placed in one of the compartments containing Murashige & Skoog agar (MS-A). Methyl Red &Voges Proskauer agar (MRVP-A), Nutrient agar (N-A) or MS-A were inoculated with the bacterial suspensions in nutrient broth. The MRVP-A, MS-A and N-A media without inoculants were used as control media. Finally, plates were sealed

with parafilm and distributed in a completely randomized design under the conditions previously described. The evaluation of *L. sativa* seedling growth was measured on day 7 and 10, according to Minerdi et al. (2011) and Gutiérrez-Luna et al. (2010), respectively.

3.2.10 Evaluation of 3H2B on *L. sativa* growth promotion. The 3H2B (Merck®) was evaluated as growth inducer through two-compartment Petri dish system (90x15 mm) with two, 2 day-old *L. sativa* seedlings placed into one of the compartments containing MS-A 0.5X. In the other compartment of the Petri dish containing the same medium, 20 µL of a compound containing 10, 1, 0.1, 0.01, 0.001 or 0.0001 mg 3H2B was applied to a sterile Whatman paper disk (distance from central partition=1 cm) (Zou et al., 2010).

3.2.11 Determination of 3H2B production and bacterial growth curve. The selected bacterial isolate (*Bacillus* sp. BCT9) showing the best agronomical parameters (i.e., foliar and radical growth) was grown in MRVP medium (Merck®) in a proportion of 1:10 (inoculums: culture medium) (in triplicate). The tubes were incubated at 28°C for 8 h with continuous shaking (160 rpm). The AB was measured each hour at 600 nm (Campos et al., 2014). Simultaneously, the production of 3H2B was evaluated through a colorimetric method, in which an aliquot of 1 mL of the bacterial suspension was centrifuged to obtain two phases (pellet and supernatant). Then, 5% α-naphthol and 40% KOH were added to the supernatant. The absorbance was measured at 540 nm (modified procedure of Xiao et al. 2012).

3.2.12 Evaluation of *Lactuca sativa* growth parameters. After inoculation, the number of lateral roots on each seedling was recorded. Both root and shoot lengths were measured through ImageJ software (National institutes of health). Finally, the dry weight of each seedling was measured after a 3-day exposure to 45°C to remove excess water.

3.2.13 Statistical analysis. Data from growth promotion experiments were analyzed by Statistix v10. Significant differences among treatments were analyzed by an ANOVA test ($p \leq 0.05$) and separated by LSD test.

3.3 RESULTS

Screening of bacterial isolates producing 3-hydroxy-2-butanone (3H2B). Soil bacteria were isolated with PCA because it is considered a universal bacterial growth medium. After the incubation period, single bacterial colonies were obtained, and the screening for 3H2B-producing bacteria was conducted using the VP test. The results showed that bacterial isolates *Serratia* sp. BCT34, *Bacillus* sp. BCT11, *Bacillus* sp. BCT21, *Staphylococcus* sp. BCT54, *Bacillus* sp. BCT4, *Bacillus* sp. BCT9, *Bacillus* sp. BCT5, *Bacillus* sp. BCT74-1, *Bacillus* sp. BCT74-2 and *Bacillus* sp. BCT53 had the ability to produce this compound (data not shown). Therefore, these isolates were selected for the subsequent step of this study *Pseudomonas* sp. BCT42 was included as the control (VP negative) (BCT= Derived from the Spanish name "Bacteria Cebem Temuco").

Identification of bacterial isolates and phylogenetic analysis. To identify bacterial isolates, 16s rRNA sequencing was used. The results indicated that the bacterial isolates belonging to the genera *Bacillus* (72.72%), *Serratia* (9.09%), *Pseudomonas* (9.09%) and *Staphylococcus* (9.09%) had high sequence similarity (Table 1). Likewise, the phylogenetic analysis of the bacterial isolates suggested the presence of four main groups (Fig. 1). The first node was formed by grouping together *Bacillus* sp. BCT74-2 and *Bacillus* sp. BCT11 and a slightly distant *Bacillus* sp. BCT9 (94%). Likewise, bootstrap analysis suggested that the strains *Bacillus* sp. BCT74-1 and *Bacillus* sp. BCT4 were grouped with 75% confidence. Moreover, it is suggested that *Bacillus* species, such as *Bacillus* sp. BCT53

and *Bacillus* sp. BCT5 have a close phylogenetic relationship (77%). Finally, Gram (-) species are clustered within group 2-3. It is worth noting that *Pseudomonas* sp. BCT42 and *Serratia* sp. BCT34 appear to be phylogenetically distant from *Staphylococcus* sp. BCT54, as well as from the other groups (Fig. 1).

Table 1. 16S identification of soil bacterial isolates through blast analysis of 16S rRNA sequences ([a] according to comparison with genbank database).

Isolates	Identification (Accession No) [a]	Similarity (%)	Accession No
BCT11	*Bacillus subtilis* strain S15B1 (KU363820.1)	99	KX395630
BCT9	*Bacillus amyloliquefaciens* strain longC (KX078078.1)	100	KX395632
BCT34	*Serratia marcescens* strain LAHAAB05 (KP830087.1)	100	KX395635
BCT74-2	*Bacillus subtilis* strain CI7 (KU681037.1)	100	KX395625
BCT42	*Pseudomonas* sp. 12Kp4 (KT825718.1)	99	KX395626
BCT5	*Bacillus megaterium* strain SA1 (KX197921.1)	99	KX395631
BCT53	*Bacillus megaterium* strain SA1 (KX197921.1)	100	KX395633
BCT54	*Staphylococcus epidermidis* strain SM 5-7 (KX267887.1)	99	KX395629
BCT74-1	*Bacillus pumilus* strain I-6 (KX 261338.1)	99	KX395627
BCT21	*Bacillus pumilus* strain I-6 (KX 261338.1)	100	KX395628
BCT4	*Bacillus pumilus* strain I-6 (KX 261338.1)	100	KX395634

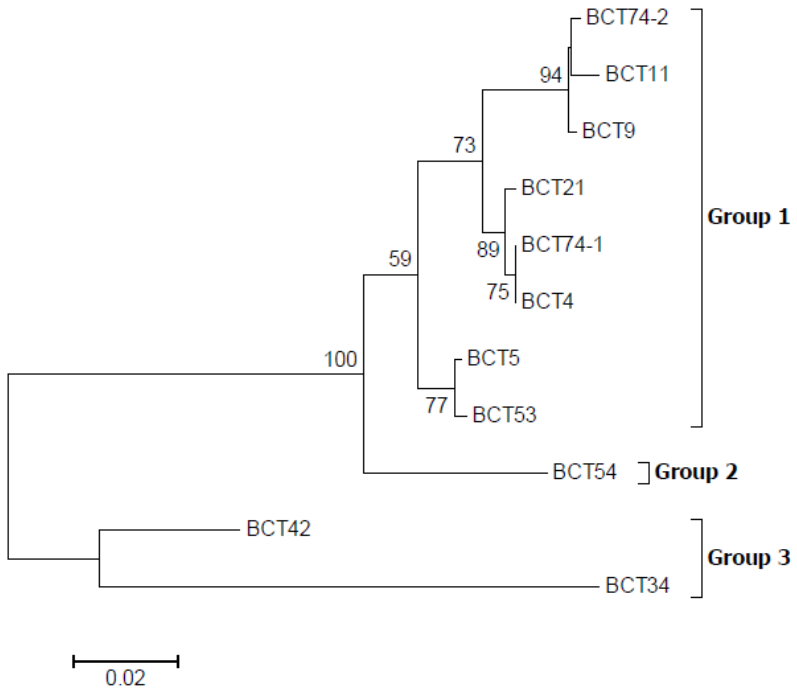


Figure 1. Phylogenetic analysis of bacterial isolates used to evaluate growth promotion activity elicited by volatile organic compounds.

Biochemical characterization of selected bacterial strains. The bacterial characterizations indicated that all strains were negative for indole production, methyl red and oxidase assays (Table 2). However, the strains tested positive in the Voges Proskauer, catalase and casein hydrolysis assays. In addition, only *Bacillus* sp. BCT9 had the ability to hydrolyze starch and liquefy gelatin. It seems that the majority of the strains did not have the ability to grow at pH 4 and in medium containing 14% NaCl (w/v), while *Bacillus* sp. BCT53 did not grow at pH 11 and 7% NaCl (w/v). All isolates grew in a temperature range from 18°C to 45°C.

Table 2. Morphological and biochemical description of soil bacterial isolates (+ = positive test; - = negative test).

Parameter	BCT4	BCT9	BCT53
Indole production	-	-	-
Red methyl	-	-	-
Voges Proskauer	+	+	+
Oxidase	-	-	-
Catalase	+	+	+
Starch hydrolysis	-	+	-
Casein Hydrolysis	+	+	+
Gelatin Liquefaction	-	+	-
Growth at			
pH 4	-	-	-
pH 7	+	+	+
pH 11	+	+	-
NaCl 7% (w/v)	+	+	-
NaCl 14% (w/v)	-	-	-
18°C	+	+	+
28°C	+	+	+
35°C	+	+	+
45°C	+	+	+

Carbohydrate metabolisms of selected bacterial isolates. The results demonstrated that different *Bacillus* strains had the ability to ferment diverse carbohydrate substrates as shown in Table 3. The *Bacillus* sp. BCT9 and *Bacillus* sp. BCT4 strains were characterized by fermenting 45.8% and 43.8% of tested carbohydrates, respectively, at the time of evaluation. However, both strains showed slight qualitative differences. In addition, *Bacillus* sp. BCT53 showed a lower ability to ferment carbohydrates than *Bacillus* sp. BCT9, amounting to 37.5% of total sugars at 48 h of evaluation.

Table 3. Carbohydrate fermentation of isolates using API 50CH strips (+ = positive reaction; - = negative reaction; +/- = medium reaction).

Substrate	*Bacillus* strains					
	BCT9		BCT53		BCT4	
	24h	48h	24h	48h	24h	48h
Glycerol	+	+	+	+	+/-	+/-
Erythritol	-	-	-	-	-	-
D-arabinose	-	-	-	-	-	-
L-arabinose	+	+	-	+/-	+/-	+
D-ribose	+	+	-	+/-	+/-	+
D-xylose	+/-	+/-	-	+/-	-	-
L-xylose	-	-	-	-	-	-
D-adonitol	-	-	-	-	-	-
Methyl-βD-xylopyranoside	-	-	-	-	-	-
D-galactose	-	-	-	-	+/-	+
D-glucose	+	+	+	+	+	+
D-fructose	+	+	+	+	+	+
D-mannose	+	+	-	-	+	+
L-sorbose	-	-	-	-	-	-
L-rhamnose	-	-	-	-	-	-
Dulcitol	-	-	-	-	-	-
Inositol	+	+	-	-	-	-
D-mannitol	+	+	+	+	+/-	+/-
D-sorbitol	+	+	-	-	-	-
Methyl-αD-mannopyranoside	-	-	-	-	-	+/-
Methyl-αD-glucopyranoside	+	+	-	-	-	+/-
N-acétylglucosamine	-	-	+	+	+/-	+/-
Amygdaline	+/-	+/-	-	-	+/-	+
Arbutine	+/-	+/-	-	-	+	+
Esculine citrate de fer	-	-	-	-	-	-
Salicine	+	+	-	-	+	+
D-celiobiose	+	+	-	-	+/-	+/-
D-maltose	+	+	+	+	-	-
D-lactose (origine bovine)	-	-	-	-	-	-
D-melibiose	-	-	-	+/-	-	+/-
D-saccharose	+	+	+	+	+	+
D-trehalose	+	+	+	+	-	+
Inuline	-	-	-	-	-	-
D-mélezitose	-	-	-	-	-	-
D-raffinose	-	+	+	+	-	+/-
Almidon	+	+	+/-	+	-	-
Glycogéne	+	+	+/-	+	-	-
Xylitol	-	-	-	+/-	-	-
Gentiobiose	+	+	-	+/-	-	-
D-turanose	-	-	-	+/-	-	+/-
D-lyxose	-	-	-	-	-	-
D-tagatose	-	-	-	-	+	+
D-fucose	-	-	-	-	-	-
D-arabitol	-	-	-	-	-	-
L-arabitol	-	-	-	-	-	-
Potassium Gluconate	-	-	-	-	-	-
Potassium 2-cétogluconate	-	-	-	-	-	-
Potassium 5-cétogluconate	-	-	-	-	-	-

Lactuca sativa growth promotion by VOCs. For the investigation of growth-inducing activity elicited by VOCs released by different bacterial isolates, a laboratory study using divided Petri dishes under laboratory conditions over 7 days was performed. Our findings showed that *L. sativa* seedlings exposed to bacterial volatiles exhibited different growth percentages depending on the bacterial strain and culture medium used. Table 4 shows the effect of bacterial volatiles in the number of lateral roots, primary root length, shoot length and dry weight. The best bacterial performance was developed by the strain *Bacillus* sp. BCT53 eliciting a significant increase in the number of *L. sativa* lateral roots when it was grown in N-A (164.7%) and MRVP-A (109.5%). *Bacillus* sp. BCT9 had the ability to induce a significant increase in the number of lateral roots when it was planted in MS-A (77.3%), N-A (100%) and MRVP-A (85.7%). Furthermore, VOCs released from the strains *Bacillus* sp. BCT4, *Bacillus* sp. BCT53 and *Bacillus* sp. BCT9 elicited a significant increase in primary root length at 153.7%, 60.7% and 57.1%, respectively, when they were grown in MS-A. Shoot length was increased in the presence of *Bacillus* sp. BCT53 (24.13%) when it was grown in MS-A. Moreover, VOCs released from strains *Bacillus* sp. BCT9 and *Bacillus* sp. BCT4 caused a slight increase in the shoot length (17.2%) when the bacteria were grown in N-A medium, and *Bacillus* sp. BCT4 grown in MRVP-A medium elicited an increase in the shoot length by 48.1%. The same three strains elicited the increase in the dry weight from 40% (MRVP-A) to 86.7% (N-A). Considering the results shown in Table 4, the *Bacillus* strains BCT53, BCT9 and BCT4 were selected for developing a longer experiment because of two main factors: a) the respective VOCs elicited the highest increase in the number of lateral roots, primary root length, shoot length and dry weight, and b) these effects were observed in all culture media.

Table 4. Effects of volatile organic compounds emitted by diverse soil isolates on lateral root number, primary root length, shoot length and dry weight on *Lactuca sativa* seedlings after 7 days of treatments applied. Letters indicate statistically significance differences according to ANOVA (LSD test) for each column (P< 0.05) (N= 15-20).

Gram	Genus	Bacterial isolates	Number of lateral root			Primary Root length (cm)			Shoot length (cm)			Dry weight (mg)		
			MS-A	N-A	MRVP-A	MS-A	N-A	MRVP-A	MS-A	N-A	MRVP-A	MS-A	N-A	MRVP-A
+	*Bacillus*	BCT11	3.6^{ab}	3.2^{bc}	3.3^{bc}	2.4^{g}	2.6^{ef}	3.1^{ef}	3.1^{bc}	3.3^{a}	3.6^{b}	1.8^{bc}	2.2^{bcd}	2.1^{cde}
		BCT21	3.1^{abcd}	3.3^{b}	2.8^{cd}	3.0^{efg}	2.4^{f}	3.7^{cde}	2.9^{bcd}	3.0^{abc}	3.0^{de}	1.7^{bc}	2.1^{cd}	2.5^{bc}
		BCT9	3.9^{a}	3.4^{b}	3.9^{ab}	4.4^{bc}	3.8^{ab}	4.1^{bc}	3.3^{bc}	3.4^{a}	3.1^{cd}	2.7^{a}	2.8^{a}	3.1^{a}
		BCT4	3.0^{bcd}	3.4^{b}	3.3^{bc}	7.1^{a}	3.7^{ab}	3.8^{cde}	3.3^{bc}	3.4^{a}	4.0^{a}	2.8^{a}	2.7^{a}	2.8^{ab}
		BCT5	2.8^{bcd}	3.2^{bc}	3.3^{bc}	2.9^{fg}	2.3^{f}	3.3^{def}	3.0^{bcd}	2.9^{bcd}	3.5^{bc}	1.6^{cd}	2.2^{cd}	2.4^{cd}
		BCT53	3.3^{abc}	4.5^{a}	4.4^{a}	4.5^{b}	3.7^{ab}	4.0^{bcd}	3.6^{ab}	3.2^{ab}	3.3^{bcd}	2.5^{a}	2.5^{ab}	2.9^{a}
		BCT74-1	1.6^{f}	2.2^{de}	2.5^{cd}	4.0^{bcd}	3.7^{ab}	4.6^{ab}	2.7^{cd}	2.9^{bcd}	3.3^{bcd}	1.6^{cd}	1.9^{d}	1.8^{ef}
		BCT74-2	2.4^{def}	2.4^{cde}	1.9^{d}	3.6^{de}	4.1^{a}	5.2^{a}	2.9^{bcd}	2.3^{e}	2.6^{e}	1.2^{d}	2.0^{d}	1.9^{ef}
-	*Pseudomonas*	BCT42	2.6^{cde}	2.9^{bcd}	2.8^{cd}	4.0^{bcd}	3.8^{ab}	4.9^{a}	2.6^{d}	2.6^{de}	3.0^{de}	1.6^{cd}	1.9^{d}	2.0^{ef}
	Serratia	BCT34	4.0^{a}	2.8^{bcd}	2.3^{d}	3.7^{cde}	3.5^{bc}	4.5^{ab}	3.1^{bc}	2.8^{cd}	3.0^{de}	2.0^{b}	2.3^{bc}	2.1^{de}
	Staphylococcus	BCT54	1.8^{ef}	1.8^{e}	2.3^{d}	3.5^{def}	3.2^{cd}	3.2^{ef}	3.8^{a}	3.1^{abc}	3.1^{de}	1.6^{cd}	2.1^{cd}	1.7^{f}
Control			2.2^{def}	1.7^{e}	2.1^{d}	2.8^{g}	3.0^{de}	2.8^{f}	2.9^{bcd}	2.9^{bcd}	2.7^{e}	1.7^{bc}	1.5^{e}	2.0^{ef}

Volatiles emitted by the three strains had the ability to induce growth on *L. sativa* after 10 days of applied treatments are shown in Figure 2. The results indicated that the number of lateral roots was increased when *Bacillus* sp. BCT4, *Bacillus* sp. BCT53 and *Bacillus* sp. BCT9 were grown in MRVP-A, and VOCs produced by *Bacillus* sp. BCT53 and *Bacillus* sp. BCT9 elicited an increase in the number of lateral roots when they were grown in MS-A and N-A media (Fig. 2b). Dry weight was significantly enhanced by the effect of VOCs released from *Bacillus* sp. BCT9 grown in all culture media (Fig. 2c). The largest shoot length increase was observed when BCT9 was grown in MRVP-A and N-A media (Fig. 2d). Finally, primary root length particularly increased by VOCs released from *Bacillus* sp. BCT9 when it was grown in N-A and MRVP-A, with similar percentages of 69% and 73%, respectively (Fig. 2e).

The 3H2B as growth inducer on *L. sativa* seedlings. A bioassay using a two-compartment Petri dish was performed to evaluate the effect of 3H2B on *L. sativa* growth parameters. Figures 3b and 3c indicated that 3H2B stimulated root length growth to 33% (0.01 mg) and number of lateral root to 130% (0.1 mg) ($P< 0.05$).

Production of 3H2B using *Bacillus* sp. BCT9 as a model. The dynamic production of 3H2B by *Bacillus* sp. BCT9 was conducted through a bacterial growth curve as indicated in Figures 3e-f. Thus, the 3H2B production was initiated from 2-4 h, reaching the highest concentration between 5-6 h, suggesting that the emission likely occurs during the lag phase and increased during early log phase of growth.

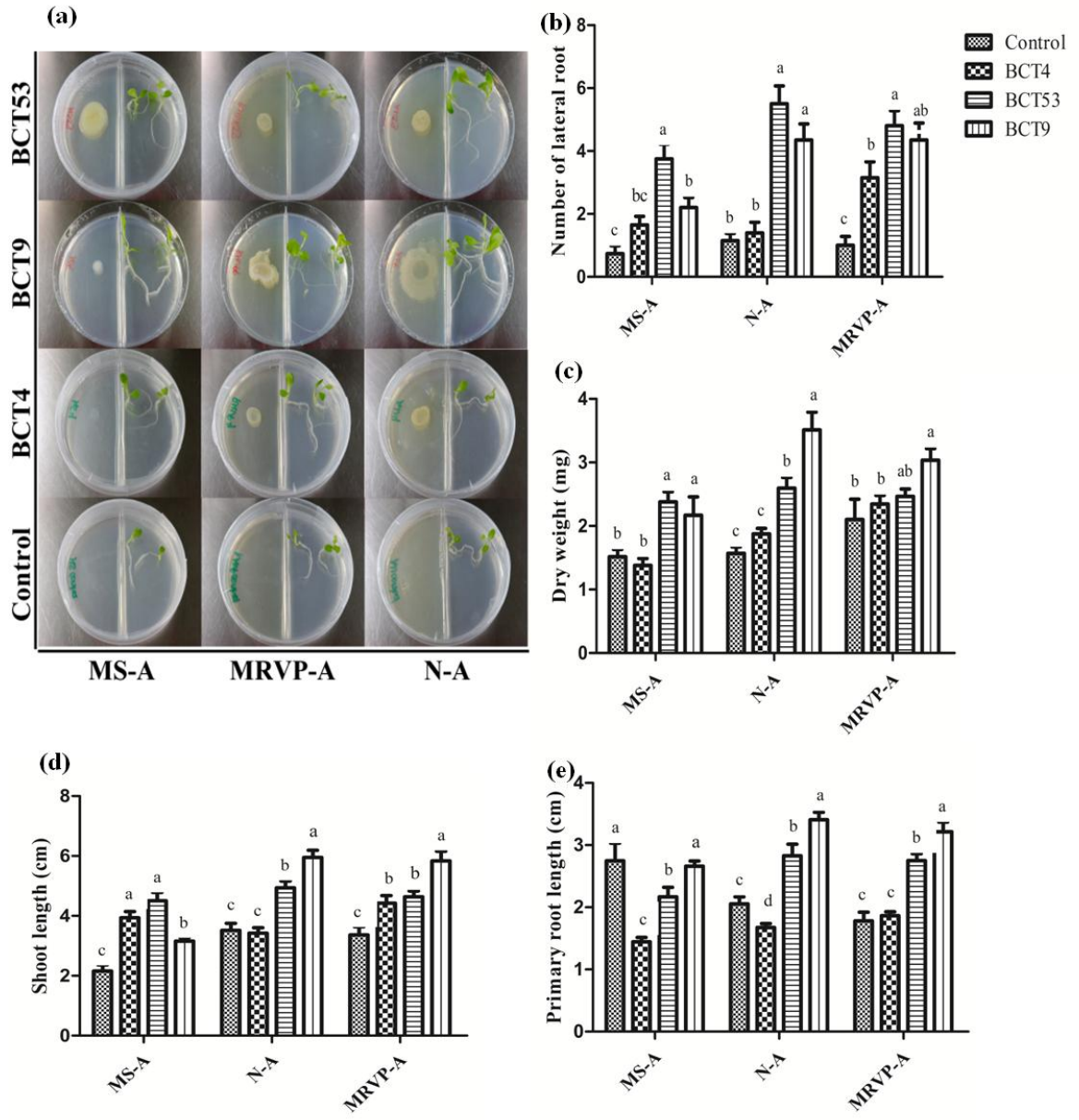


Figure 2. Bacterial volatiles as growth inducers on *L. sativa* on day 10 (a) The effects of bacterial volatiles compounds released from BCT4, BCT9 and BCT53 on (b) number of lateral root, (c) dry weight, (d) shoot length and (e) primary root length when the isolates grown in N-A, MS-A and MRVP-A after applied the treatments. Letters indicate statistically significant differences according ANOVA test (LSD; $P < 0.05$) (N=15-20). Control: non-inoculated seedlings. Bars indicate error standard.

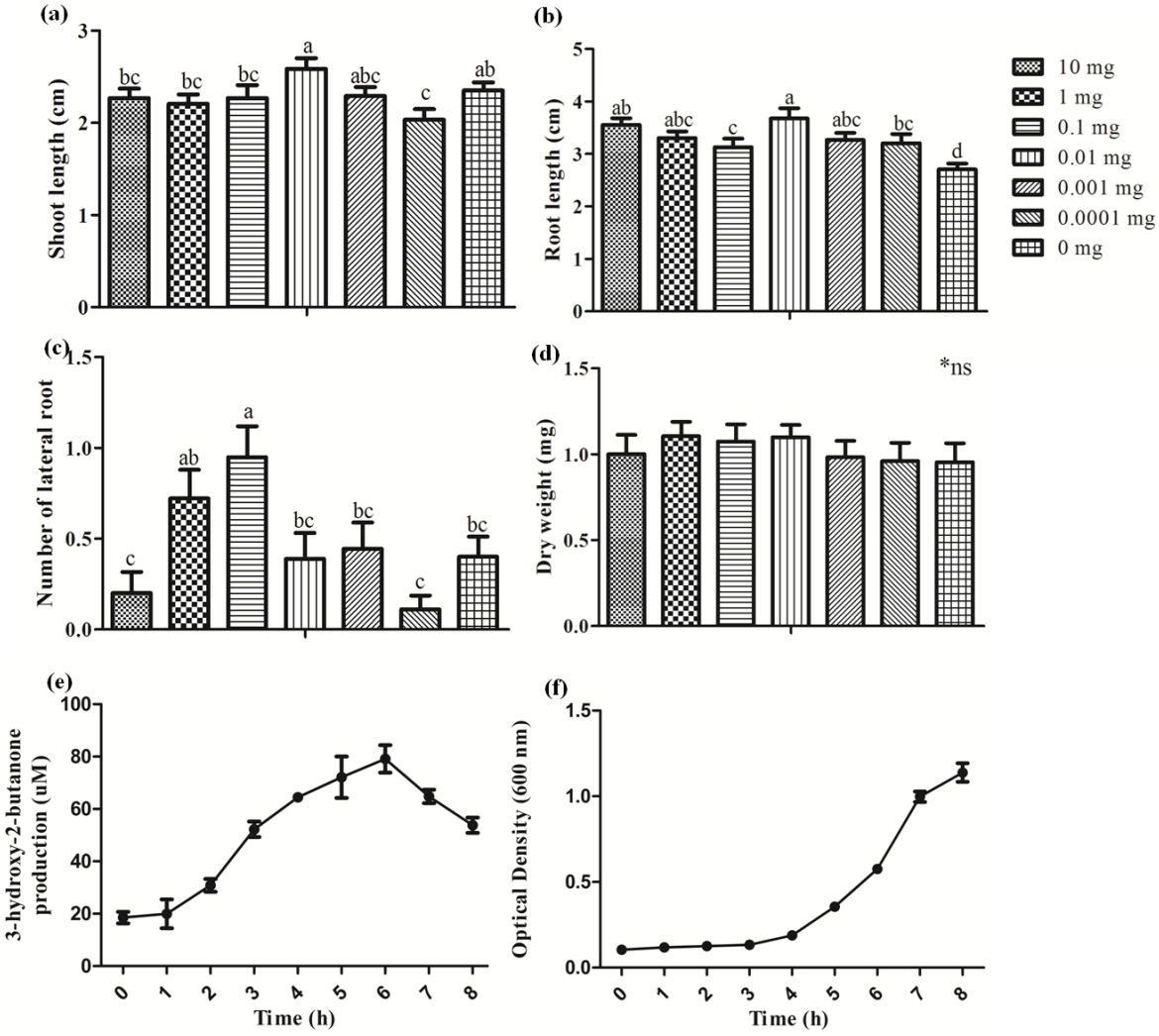


Figure 3. The 3-hydroxy-2-butanone (3H2B) as growth inducer on *L. sativa* growth. (a) The effects of 0.0001 mg, 0.001 mg, 0.01 mg, 0.1 mg, 1 mg and 10 mg to modulate *L. sativa* growth. The effect of 3H2B on (a) shoot length, (b) primary root length, (c) number of lateral root and (d) dry weight on 10-day-old *L. sativa* seedlings. (e-f) The 3H2B production versus bacterial growth. Letters indicate statistically significant differences according to ANOVA test (LSD; N= 18-20; P< 0.05). * indicate there is no statistically significant difference. Bars indicated standard error.

3.4 DISCUSSION

The rhizosphere comprises a soil zone with intensive association of bacterial species with plant roots and contains a higher density of bacterial species. These bacteria have significant effects on plant growth, with abilities to produce non-volatile compounds that induce growth (Van Loon, 2007; Bulgarelli et al., 2013). Moreover, recent studies have reported that VOCs emitted by some bacterial strains have the capacity to induce growth on plant target involving only aerial contact; VOCs have the ability to act as aerial signal molecules inducing plant growth (Bitas et al., 2013; Chung et al., 2015). Currently, some bacterial species (i.e. *Bacillus*, *Pseudomonas* and *Arthrobacter*) have been described for their capacity to release bioactive VOCs that induce growth in *A. thaliana*. However, little information about the application in vegetable species is available, which encourages the development of new, rapid and efficient screening methods to search novel bacterial compounds that induce growth. Our results propose to the VP biochemical test to be used as a bacterial strain selection strategy because it is a simple, fast and easy technique for 3-hydroxy-2-butanone (3H2B) detection, a compound described as growth inducer and elicitor of systemic resistance on *A. thaliana* (Ryu et al., 2003; Rudrappa et al., 2010). This test allowed us to select 10 bacterial strains belonging to *Bacillus*, *Serratia* and *Staphylococcu*s genus, which is consistent with findings from previous studies (Marquez-Villavicencio et al., 2011; Filipiak et al., 2012; Sun et al., 2012).

In the next stage of the experiment, we evaluated the VOCs emitted by the selected bacterial strains inoculated in three culture medium conditions. MRVP-A medium was used to enhance the production of 3H2B and 2,3-butanediol. MS-A has been utilized in several previous reports as a medium for bacteria growth, and N-A has been used in studies

involving *M. sativa* growth (Ryu et al., 2003; Blom et al., 2011; Velázquez-Becerra et al., 2011). Our results showed the significant and different abilities of volatiles released by *Bacillus* strains to increase *L. sativa* growth depending on the culture medium used; probably their differential composition or complexity can modulate the activation of metabolisms pathways, generating a divergent response in the emission of volatile profile. It is worth noting that *Bacillus* sp. BCT4, *Bacillus* sp. BCT9 and *Bacillus* sp. BCT53 had the capacity to increase parameters evaluated independently of the culture media used, while other strains, such as *Bacillus* sp. BCT74-2 and *Bacilus* sp. BCT74-1, were dependent on specific conditions to stimulate *L. sativa* growth. The differential effects elicited by volatiles emitted by the strains belonging to *Bacillus* could be explained by the divergence of the volatile profiles in species of the same genus. This is a hypothesis supported by the studies of Farag et al. (2006) and Ryu et al. (2003), which indicated that *B. subtilis* GB03 and *B. amyloliquefaciens* IN937a produce different volatile compounds with specific action mechanisms to stimulate growth in *A. thaliana*. To date, these specific mechanisms are unknown in *L. sativa* seedlings. However, exhaustive studies on *A. thaliana* have shown that VOCs induce changes at the metabolic, growth, stress and signaling levels (Zhang et al. 2007, 2008, 2009).

Additionally, our findings suggest that the BCT34 strain, belonging to the genus *Serratia*, increased primary root length independently of culture media used, while specific conditions were needed to increase dry weight and number of lateral roots. These results coincide with a stimulatory effect of VOCs on the fresh weight of *A. thaliana* emitted by *S. odorifera* 4Rx13, as reported by Kai and Piechulla (2009). In contrast, Vespermann et al. (2007) reported that VOCs emitted by *Serratia* species reduced growth in *A. thaliana*.

Moreover, BCT42, belonging to *Pseudomonas* genus, only had a significant effect on the primary root length and this was independent of culture media. Despite our findings, *Pseudomonas* genus has been previously characterized by its ability to inducing shoot length, according to the reported by Santoro et al. (2011). This study indicated that volatiles released by *P. fluorescens* act as growth inducers on peppermint, increasing root dry weight, shoot fresh weight and leaf surface area. Likewise, volatiles emitted by *P. fluorescens* strain SS101 increased tobacco fresh weight under laboratory conditions (Park et al., 2015). Finally, the BCT54 strain belonging to *Staphylococcus* genus did not elicited the growth of *L. sativa,* in contrast to growth effects reported by Vespermann et al. (2007) in *A. thaliana*.

The specific interaction between bacterial strain and plant target is highly important to regulate the growth. Moreover, the results showed that the culture media is essential for the emission of the determinate VOC mixture. For instance, our results show that the type of the culture media, such as MRVP-A (containing glucose as carbon source; pH 6.9±0.2), N-A (composed by beef extract and peptone; pH 6.8 ±0.2) and MS-A (consisting of mineral nutrients with a sucrose as C source and lower pH; pH 5.7), can affect the volatile organic compound production. *Bacillus* sp. BCT53, *Bacillus* sp. BCT9 and *Bacillus* sp. BCT4, independent of the culture condition, had the highest capacity to induce growth on evaluated parameters after 7 days of treatment. Therefore, these strains were selected to perform a second evaluation on day 10, showing a divergence of effects by *Bacillus* strains on *L. sativa* growth and suggesting that the evaluation time can be an important factor to determine the effects of the VOCs. In first instance the evaluation was performed on day 7 to tracing-volatile producing bacteria with capacity to induce *L. sativa* growth. Minerdi et

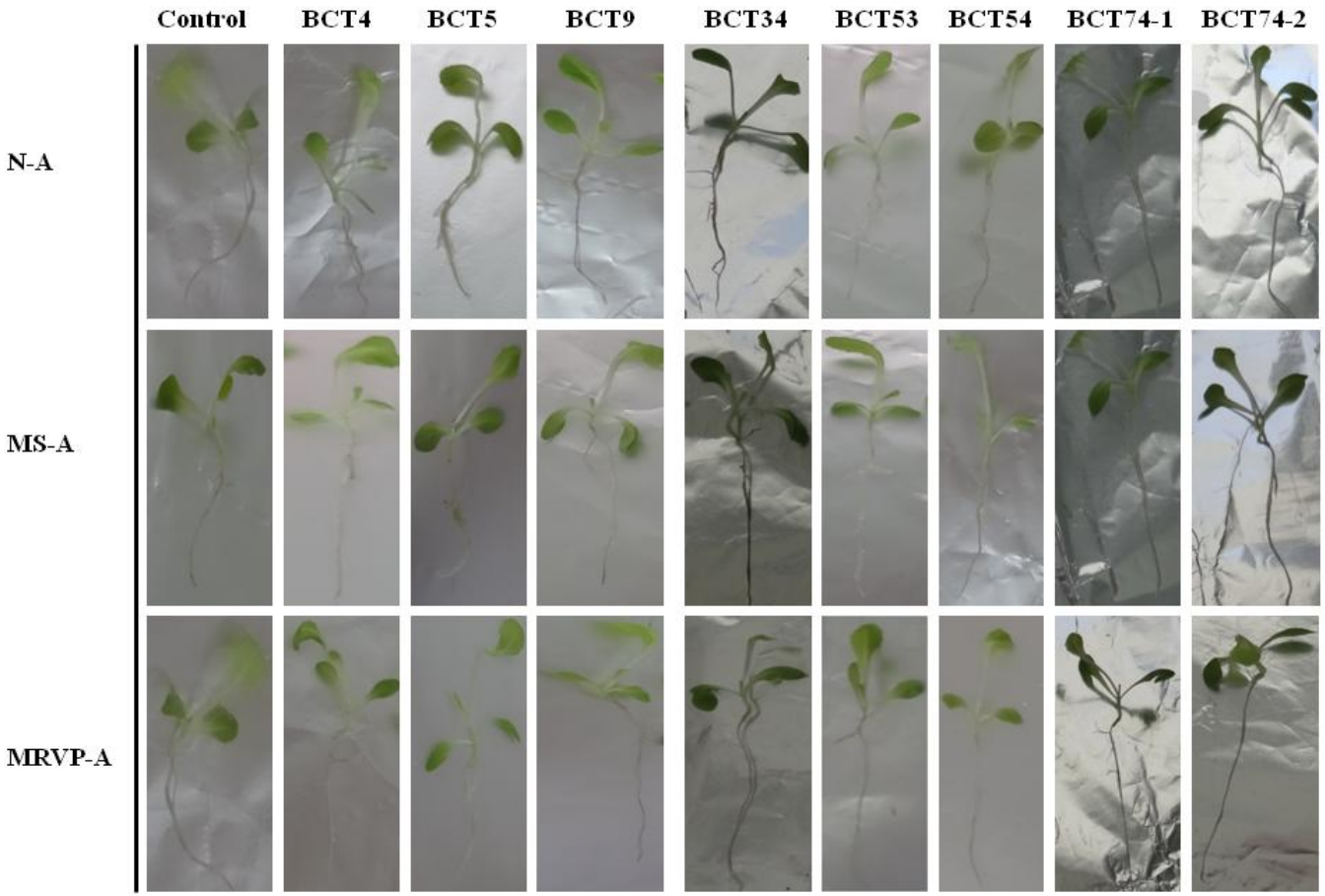
al. (2011) detected growth induction when *L. sativa* seedlings were exposed for 7 days to volatiles emitted by *Fusarium oxysporum* MSA35, showing that gene expression of expansin was regulated at root and shoot level. This study supported that this period is sufficient to detect changes on growth and physiological parameters on seedlings. Furthermore, the same exposition period was used by Zhang et al. (2007) to detect changes after *A. thaliana* exposed to volatiles release by *B. subtilis* GB03, showing that genes related to metabolism, growth, stress and signaling are regulated. Besides, Ann et al. (2013) evaluated volatile effects released from *Bacillus vallismortis* EXTN-1 on tobacco growth, showing that the impact of culture media for bacterial growth is a critical parameter to evaluate the ability to release volatile as growth inducers and Zou et al. (2010) determined the increase of fresh weight on *A. thaliana* after exposition of volatiles released from *B. megaterium* XTBG34 from the seventh day. In the second instance, we used 10 days to measure the ability of selected bacterial strains to release volatile compounds for eliciting growth. In the study performed by Ryu et al. (2003), *A. thaliana* seedlings were exposed to volatiles release by *B. subtilis* GB03, detecting the increase of leaf surface area after 10 days of exposition. Besides, Gutiérrez-Luna (2010) reported that changes at radical level, including lateral root length, lateral root number and primary root length were found in *A. thaliana* after exposition to volatiles release from *Bacillus* strains, suggesting that important effects derived from volatile compounds can be found in this evaluation time. Therefore, short periods of analysis allow determining changes at root and leaf level after exposure to volatiles, which produce changes in gene regulation that lead to physiological changes. The quick action of volatiles gives an early advantage in seedlings due to the early strengthening of seedlings to survive and established in soil environment, allowing avoid large losses of seedlings designed for food production. Additionally, the characterization of

selected strains *Bacillus* sp. BCT9, *Bacillus* sp. BCT4 and *Bacillus* sp. BCT53 were different on enzymatic and physiological parameters (% NaCl, pH and T°) and carbohydrate fermentation. Hence, these strains can emit volatiles under different conditions to resist abiotic stress, but more studies are required.

Bacteria strains used in this research were selected according their ability for producing 3H2B. Therefore, in a second experiment the capacity of this compound to affect plant growth was investigated. The results showed that 3H2B elicited a stimulatory effect on primary root length and the numbers of lateral roots, suggesting that other volatiles released by the bacterial strains may induce an increase in shoot length and dry weight of *L. sativa*. Finally, *Bacillus* sp. BCT9 was used as a model to determine 3H2B concentration along with bacterial growth, given its greater ability to induce growth in *L. sativa* at 7 and 10 days. The results indicated that 3H2B is emitted mainly in early stage of growth, due to either a formation of 2,3-butanediol in the late stage or a decrease in glucose concentration (substrate precursor) at the point that 3H2B production is stopped. One aspect to consider about the effect of volatile organic compounds on growth induction may be their degree of volatility. It is noteworthy that the degree of volatility of a determinate compound has a close relation with the vapor pressure. 3H2B is chemically characterized by having 1.9 ± 0.6 mm Hg of vapor pressure at 25°C. Furthermore, β-caryophyllene has been reported by Minerdi et al. (2011) as growth inducer on *L. sativa* seedlings while α-humulene had no growth promoting effect under the similar conditions, both have a vapor pressure of 0.0 ± 0.3 mm Hg at 25°C, suggesting that the effect depend on the structure of the bioactive compound. In addition, in this study 3H2B showed their ability to induce growth activity, independent of its higher vapor pressure. The above suggests that the growth effect given

by a volatile organic compound depend on ability of a single compound to elicit cellular signals that trigger physiological changes, independent of their volatility.

In conclusion, this study provides clear evidence that a conventional biochemical test can be used as a rapid strategic tool for investigating bacterial species strains as novel volatile sources. Moreover, we showed that VOC perception by the plant host can generate different responses, depending on the specific bacterial strain-plant seedling interaction, and can trigger changes to stimulate growth at the root and shoot level in *L. sativa* seedlings. Additionally, enzymatic and physiological tests can be used as new strategies to monitor VOCs sources for inducing tolerance or resistance against environment stresses, constituting an important alternative to apply VOCs in agriculture. Therefore, this study suggests that bacterial VOCs can be used as an environmentally friendly alternative to reduce the application of agrochemicals, potentiating its use as natural product in horticulture.



Supplementary Figure 1. Effects of volatile organic compounds emitted by diverse bacterial strains on *L. sativa* seedlings on day 7.

CHAPTER IV:

Volatile organic compounds stimulate plant growing and seed germination of *Lactuca sativa*

4.1. INTRODUCTION

Volatile organic compounds (VOCs) are molecules with low molecular weight (300 g mol^{-1}) and high vapour pressure (0.01 kPa at 20°C) that include diverse chemical compounds (i.e. ketones). In the last decade, VOCs emitted by species belonging to *Bacillus* genus have been described for their ability to induce growth in *Arabidopsis thaliana*, which is usually used as model plant (Ryu et al., 2003; Kanchiswamy et al., 2015ab). The VOCs have the ability to elicit plant growth in absence of physical contact through the induction of physiological changes depending on doses and culture medium for bacterial growth (Zhang et al., 2007; Blom et al., 2011). Therefore, we propose that VOCs emitted by *Bacillus* species can be a new strategy to induce growth on horticultural species for reducing agrochemical products. Based on the above mentioned, *Lactuca sativa* emerges as a model vegetable to test volatiles as growth inducer due to easy management, fast germination and sensitivity to compounds exposition (Charles et al., 2011). The objectives of the present study were: (1) to evaluate culture conditions of *Bacillus* sp. BCT9 for producing volatiles with growth-inducing activity and (2) to determine the effects of identified volatile organic compounds on *L. sativa* germination.

4.2 MATERIALS AND METHODS

4.2.1 Bacterial isolates and plant growth conditions. The *Bacillus* sp. BCT9 (Genbank access number: KX395632) was routinely streaked on Plate Count Agar. Commercial seeds of *L. sativa* (Green lettuce cv Reina de mayo asepo, semillas Fito, S.A) were surface-sterilized during 8 min with 3% sodium hypochlorite and washed with sterile distilled water. Later, seeds were placed on Murashige and

Skoog basal medium with vitamins 0.5X (PhytoTechnology Laboratories, LLC™) containing 0.8% agar and 1.5% sucrose (MS-A). Petri dishes were placed under 16/8-h light-dark cycle at 20-25°C. Germinated seedlings were transferred to two-compartment Petri dishes after 2 days for experimental uses (Ryu et al., 2003).

4.2.2 Evaluation of *Bacillus* sp. BCT9 dose on *L. sativa* growth. According to the methodology reported by Fincheira et al. (2016a), bioassays were performed in two-compartment Petri dishes (90 x 15 mm) with two 2-day-old *L. sativa* seedlings placed into one of the compartments containing MS-A and the second compartment containing Nutrient agar (N-A), Methyl Red & Voges Proskauer agar (MRVP-A) or MS-A. The second compartment was inoculated with 15, 30 or 60 µL of *Bacillus* sp. BCT9 ($2x10^8$ CFU mL^{-1}). The plates were distributed in a randomized design and non-inoculated plates were used as control (Blom et al., 2011; Velázquez-Becerra et al., 2011). The evaluation of *L. sativa* growth was measured on day-10.

4.2.3 GC-MS analysis of volatile organic compounds release by *Bacillus* sp. BCT9. Bacterial isolate (25 mL) was grown in 250 mL of MRVP for 19 h at 34°C to collect volatiles using Solid Phase Micro Extraction (SPME) fiber (PDMS/DVB), previously conditioned with helium for 10 min at 250°C. Volatiles were desorbed at 250°C for 2 min in an injector of gas chromatograph coupled with mass spectrometer (Thermo Electron Corporation). The chromatographic separation was performed by DB-1 column using helium flow (1.0 mL min^{-1}). Mass spectra were acquired in the mass using 35- 500 a.m.v. electronic imput at 70 eV. The VOCs were identified by comparing Kovats indices (KIs) with the corresponding commercial standards by injecting an alkane series (C9–C26) (Tampe et al., 2016).

4.2.4 Germination toxicity assays. The disinfected seeds (n=15) were placed on the surface of MS-A in one of the compartments of two-compartment Petri dishes and exposed to different doses (50, 000 ppm, 50 ppm and 0.05 ppm) of identified volatile organic compounds placed in the other compartment during germination stage. Three plates were used to test each combination (compound x concentration). Root elongation (RE) ($RE=Elongation_{sample}- Elongation_{control}/ Elongation_{control}$) and seed germination (SG) ($SG=Germination_{sample}- Germination_{control}/ Germination_{control}$) were evaluated as toxicity parameters. The indices are designed with values in a range from -1 (maximum phytotoxicity) to >0. The toxicity effect was evaluated after 120 h according to the following scale: (1) 0 to -0.25 = low toxicity, (2) -0.25 to -0.5= moderate toxicity, (3) -0.5 to -0.75= high toxicity and (4) -0.75 to -1= very high toxicity. A value more than zero indicate growth stimulation (Bagur-González et al., 2010).

4.2.5 Statistical analysis. The growth parameters were analyzed by Statistix v10. The difference resulted from the VOCs treatments on *L. sativa* growth parameters was analyzed using analysis of variance (ANOVA) and LSD test ($P \leq 0.05$).

4.3 RESULTS

4.3.1 The effects of different culture conditions on *Bacillus* sp. BCT9 to release VOCs as growth modulator on *L. sativa*. Figure 1 shows that seedlings exhibits 24, 54, 51 and 190% increase in root length, dry weight, shoot length and number of lateral roots, respectively, when BCT9 (30 µL) was grown in N-A. Nevertheless, the exposition of seedlings to BCT9 VOCs (60 µL) grown in MRVP-A shows increased root length (80%).

L. sativa seedlings showed a 98 and 58% increase in dry weight and shoot length, respectively. Furthermore, the number of lateral roots increased more than 6-fold compared with the control. In addition, dry weight and number of lateral roots increased 127 and 95%, respectively when BCT9 (60 µL) was cultivated in MS-A. For the next stage, the MRVP medium was chosen to identify VOCs emitted by BCT9 due to its highest capacity to elicit an increase of root length.

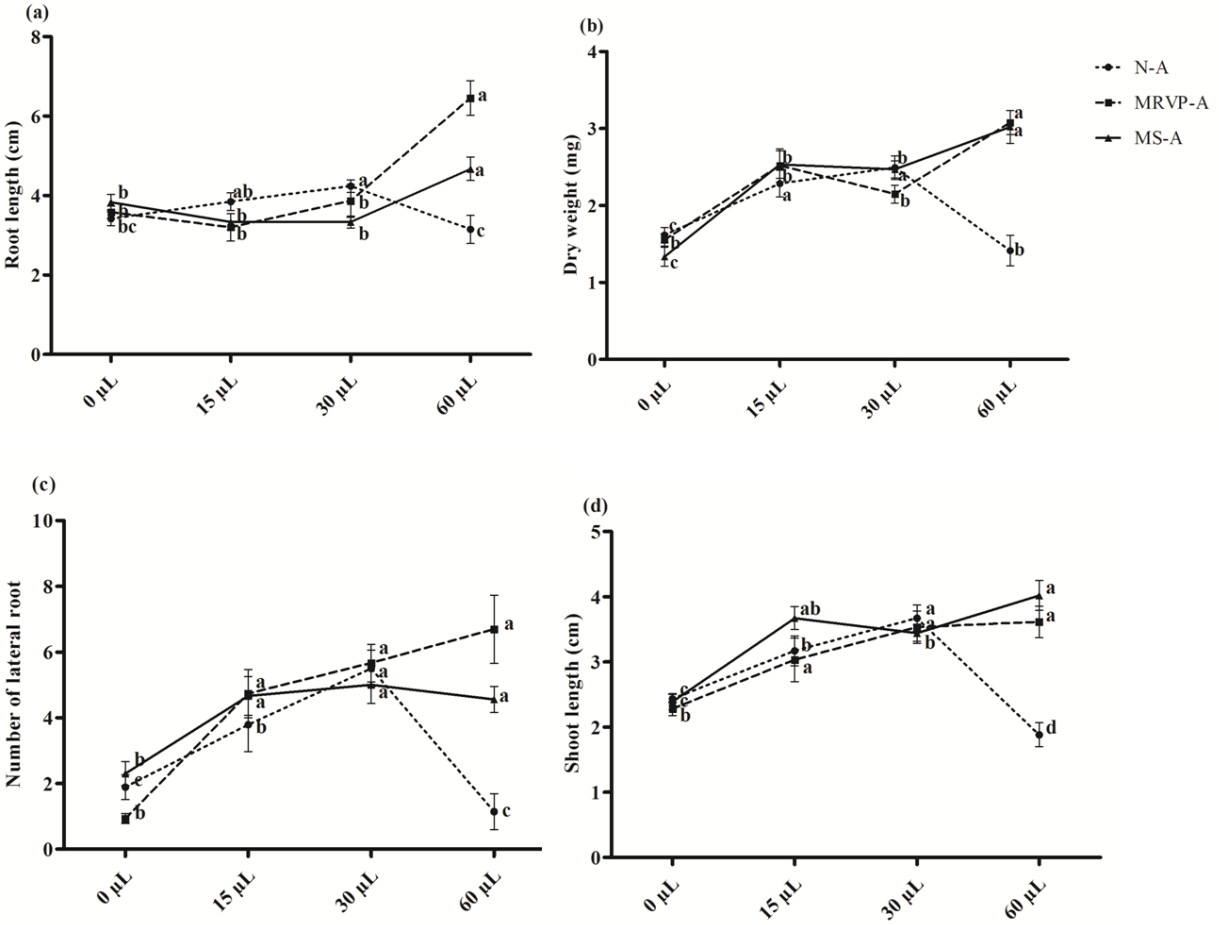


Figure 1. Effects of different doses of *Bacillus* sp. BCT9 on the emission of volatile organic compounds on growth modulation of *Lactuca sativa*. N-A= Nutrient agar; MRVP-A = Methyl Red & Voges Proskauer agar; MS-A = Murashige & Skoog agar. Bars represent the standard error. Letters indicate means that differ significantly according to ANOVA (LSD test) for each culture medium ($P < 0.05$) (N= 15-20).

4.3.2 The effect of VOCs release by BCT9 on seeds germination of *L. sativa*. The identified compounds released from BCT9 grown in Methyl Red & Voges Proskauer were 3-hydroxy-2-butanone, 2,3-butanediol, 2-nonanone, 2-undecanone, 2-tridecanone and 2-pentadecanone. The VOCs showed a low toxicity on *L. sativa*, according to indices from -0.27 to 0, only 2-nonanone at 50,000 ppm presented toxicity. Ketone compounds increased both RE and SG in at least one applied concentration. Finally, it noteworthy that 2-nonanone (0.05 ppm), 2-undecanone (50 ppm) and 2-tridecanone (0.05 and 50 ppm) stimulated the growth on *L. sativa* at germination stage (Table 1).

Table 1. Identified volatile organic compounds release from *Bacillus* sp. BCT9 cultivated in MRVP medium and their effects on germination (RT = retention time, RL= root length, Germ= germinated seeds, RE=root elongation, SG= seed germination). Letters indicate means that differ significantly according to ANOVA (LSD test) (N=3).

Compound	RT (min)	Doses (ppm)	RE(cm)	Germ (%)	RE	SG
3-Hydroxy-2-butanone	3.9	50,000	0.46 ± 0.17 ^{ef}	97	-0.16	-0.01
		50	0.49 ± 0.16 ^{ef}	90	-0.10	-0.08
		0.05	0.48 ± 0.16 ^{ef}	97	-0.12	-0.01
2,3-Butanediol	5.1	50,000	0.44 ± 0.14 ^{f}	95	-0.2	-0.03
		50	0.46 ± 0.15 ^{ef}	97	-0.16	-0.01
		0.05	0.50 ± 0.16 ^{def}	98	-0.09	0
2-Nonanone	13.0	50,000	0.40 ± 0.15 ^{f}	23	-0.27	-0.76
		50	0.59 ± 0.23 ^{bc}	95	0.07	-0.03
		0.05	0.57 ±0.19 ^{bcd}	98	0.03	0
2-Undecanone	18.7	50,000	0.61 ± 0.19 ^{b}	87	0.10	-0.11
		50	0.69 ± 0.24 ^{a}	98	0.25	0
		0.05	0.53 ± 0.18 ^{cde}	80	-0.03	-0.18
2-Tridecanone	23.9	50,000	0.63 ± 0.23 ^{ab}	90	0.14	-0.08
		50	0.60 ± 0.17 ^{bc}	98	0.09	0
		0.05	0.59 ± 0.18 ^{bc}	98	0.07	0
2-Pentadecanone	28.6	50,000	0.48 ± 0.22 ^{ef}	98	-0.12	0
		50	0.43 ± 0.16 ^{f}	83	-0.21	-0.15
		0.05	0.62 ± 0.20 ^{ab}	93	0.12	-0.05

4. 4 DISCUSSION

Bacterial species have been intensively studied for their ability to increase plant growth by emission of non-volatile compounds. In the last decade, Ryu et al. (2003) reported that VOCs can elicit growth through the activation of physiological pathways. Nevertheless, culture conditions for bacterial growth have an essential role for the emission of volatiles (Blom et al., 2011). In this study, different culture conditions of *Bacillus* sp. BCT9 were evaluated for producing VOCs as growth modulators on *L. sativa*. The VOCs produced by BCT9 elicited *L. sativa* growth depending strongly on inoculated doses, in agree with the reports by Velázquez-Becerra et al. (2011) and Blom et al. (2011), who indicated that low doses of inoculums elicit growth and high doses can induce phytotoxicity in seedlings. The same bacterial genus was studied by Asari et al. (2016), who showed that VOCs released by *Bacillus* species induced a significant increase on dry weight of *A. thaliana* (phyllosphere) when the strains grow in Luria Broth Agar (LB-A), Minimal Medium (M9) or Trypticase Soy Agar, indicating that doses of *Bacillus amyloliquefaciens* UCMB5113 from 20 to 100 µL inoculated in MS-A increased dry weight (phyllosphere). Besides, Blom et al. (2011) reported that *Burkholderia pyrrocinia* Bcc171 increased dry weight on *A. thaliana* when grown in LB-A and MRVP-A, reaching the best yield with 10 µL of applied inoculums. Furthermore, *Medicago sativa* - *Arthrobacter agilis* UMCV2 interaction was studied by Velázquez-Becerra et al. (2011), who reported a dose-dependence response of *M. sativa* exposed to VOCs release by *A. agilis* UMCV2, reaching the best increase on root length, root density, stem length and fresh weight with 50 µL of inoculum grown in N-A compared with doses from 100 to 500 µL. The studies described above suggest that low inoculums amount (range from 10 to 50 µL) have a great effect to induce growth. The

BCT9 grown in MRVP, showed a high ability to induce *L. sativa* growth, so this medium was selected to identify VOCs. The VOCs released are derived from two metabolic pathways: piruvate fermentation (3-hydroxy-2-butanone and 2,3-butanediol) and fatty acid cycle (2-nonanone, 2-undecanone, 2-tridecanone and 2-pentadecanone). Respect to germination assays, the results indicated that both indices showed a sensitivity to evaluate VOCs phytotoxicity and compounds showed a low toxicity on *L. sativa* during germination stages. Remarkably, the results indicated that 2-nonanone, 2-undecanone and 2-tridecanone had the ability to stimulate seed germination, suggesting their important influence in this stage, but more studies should be performed. This research shows the importance of culture conditions to prospect non-toxic VOCs as growth inducers in horticultural species to study VOCs as a new strategy to reduce agrochemical application. VOCs released by *Bacillus* sp. BCT9 act as growth inducer agents at shoot and root level on *L. sativa*, obtaining the best yield with exposition to VOCs released in a range from 30 to 60 µL of inoculums depending on culture conditions. In addition, the results suggest that VOCs have low toxicity effect on seeds and ketone compounds have stimulating effect on germination stage.

CHAPTER V:

Volatiles emitted by *Bacillus* sp. BCT9 act as growth modulating agents on *Lactuca sativa* seedlings

5.1 INTRODUCTION

The food is a relevant topic given the need for increasing their production and quality. In this context, chemical products are intensively applied, but their negative environmental effects and consumer preferences for chemical-free products have led to the search for novel bio-products (Jacobsen et al., 2013). An emerging alternative strategy involves the use of bacterial species that release non-volatile compounds that induce plant growth involving phytohormones, organic acids, antibiotics and siderophores (Vejan et al., 2016). In the past decade, a new mechanism mediated by volatile organic compounds (VOCs) that induce plant growth has been reported (Ryu et al., 2003; Xie et al., 2009). Hundreds of VOCs produced via plant and microbial metabolism are characterized by low molecular weights (<300 g mol^{-1}), high vapor pressures under normal conditions (0.01 kPa or higher at 20°C) and low polarity (Schulz and Dickschat, 2007; Audrain et al., 2015; Bennet and Inamdar, 2015; Kanchiswamy et al., 2015ab; Widhalm et al., 2015). Microbial volatile profiles consist of compounds possessing diverse chemical natures such as alkanes, alkenes, alcohols, esters, ketones and terpenoids, among others, and these profiles are strongly dependent on culture conditions (i.e. the culture medium) and bacterial concentrations under controlled conditions (Kai et al., 2016).

Some bacterial species release VOCs that possess the ability to modulate "plant target" growth in the absence of physical contact (Chung et al., 2015; Fincheira et al., 2016a; Kai et al., 2016). Diverse studies have focused on *Bacillus* as a VOC source to elicit the growth on *Arabidopsis thaliana*. In one study, VOCs from *Bacillus subtilis* GB03 and *B. amyloliquefaciens* IN937 increased the total surface area of *A. thaliana* (Ryu et al., 2003). According to Zhang et al. (2008), *B. subtilis* GB03 VOCs increased chlorophyll content

(88%). Later, Xie et al. (2009) reported increased fresh weight (58%) and dry weight (71%) due to VOCs emitted by the same bacteria. Furthermore, in a study by Banchio et al. (2009), GB03 increased leaf area, shoot fresh weight and root fresh weight in *Ocimum basilicum*, while Gutiérrez-Luna et al. (2010) reported the ability of some *Bacillus* strains to increase root growth in the same species. Later, Ann et al. (2013) indicated VOCs released by *B. vallismortis* EXT-1 elicited increases in the fresh and dry weights of tobacco following 7 days of exposure. More recently, Asari et al. (2016) reported an increase in the phyllosphere dry weight of *A. thaliana* exposed to volatiles emitted by *B. amyloliquefaciens* strains over a period of 18 days. These effects have been associated with the emission of VOCs such as 2,3-butanediol, 2-pentylfuran and dimethyl disulfide (Ryu et al., 2003; Zou et al., 2010; Meldau et al., 2013).

Bioactive VOCs exert different effects, such as the induction or inhibition of plant growth, depending on bacterial concentrations and culture media (Blom et al., 2011; Velázquez-Becerra et al., 2011; Asari et al., 2016). However, studies have focused on the single application of a bacterial concentration grown in a defined culture medium, i.e., Trypticase Soy agar, Methyl Red & Voges Proskauer, Nutrient Agar and Murashige & Skoog (Ezquer et al., 2010; Zou et al., 2010; Asari et al., 2016). We propose that *Bacillus* culture conditions play essential roles in the production of VOCs that induce growth, suggesting the application of such compounds to horticultural species may reduce the use of agrochemicals. *Lactuca sativa* is an adequate vegetable model to investigate the effects of VOCs given its easy management, rapid growth, and uniform germination under various environmental conditions. Moreover, this species has previously been employed to determine the phytotoxicity effects of both pure compounds and complex mixtures (Díaz de

Villegas et al., 2011; Charles et al., 2011). The objectives of the present study were (1) to evaluate the effects of *Bacillus* sp. BCT9 culture conditions on VOC emissions to promote *L. sativa* growth and (2) to identify the specific VOCs involved.

5.2 MATERIALES AND METHODDS

5.2.1. Bacterial isolate and growth conditions. *Bacillus* sp. BCT9, which was identified by 16S ribosomal sequencing, is available in GenBank under accession number KX395632. In this study, BCT9 was used to perform bioassays due to its significant growth-inducing effects on *L. sativa* seedlings through the production of volatiles, as shown by Fincheira et al. (2016a). The BCT9 strain was stored in peptone broth containing 15% glycerol at -20°C. Bacteria were grown routinely on Plate Count Agar (PCA) (Difco® Laboratories, Detroit, MI) for 2 days at 28°C under dark conditions. Single colonies were transferred to 4 mL of Nutrient broth and grown overnight with shaking at 160 rpm.

5.2.2 Chemicals and culture media. Chemicals (2,3-butanediol, 2-nonanone, 2-undecanone, 2-tridecanone and 2-pentadecanone) were obtained from Sigma–Aldrich (Steinheim, Germany). Additionally, 3-hydroxy-2-butanone, water and hexane were purchased from Merck Millipore (Darmstadt, Germany). *Bacillus* sp. BCT9 was grown on PCA (5 g of pancreatic digest of casein, 2.5 g of yeast extract, 1 g of dextrose and 15 g of agar/l) (Difco® Laboratories, Detroit, MI). Nutrient medium (3 g of beef extract and 5 g of peptone) (Difco® Laboratories, Detroit, MI) and Red Methyl & Voges Proskauer medium (7 g of peptone, 5 g of glucose, 5 g of dipotassium phosphate) (Merck Millipore, Steinheim, Germany) were supplemented with 15 g of agar/L.

5.2.3 Plant material and growth conditions. Commercial *L. sativa* seeds (Green lettuce cv Reina de mayo asepo, semillas Fito, S.A) were used. Seeds were surface-sterilized (8 min with 3% sodium hypochlorite) and washed with sterile distilled water. Then, seeds were placed on the surfaces of Petri dishes containing Murashige and Skoog basal medium containing 0.5X vitamins (PhytoTechnology Laboratories, LLC™), 0.8% agar and 1.5% sucrose (MS-A). Petri dishes were placed in a conditioned room with a 16/8-h light-dark cycle under 36-W fluorescent light. The temperature was maintained between 20-25°C. Germinated seedlings were transferred after 2 days to divided Petri dishes containing the same culture medium for bioassays (Ryu et al., 2003; Minerdi et al., 2011).

5.2.4. Evaluation of the effects of different *Bacillus* sp. BCT9 cell densities on *L. sativa* growth. Bioassays were performed in two-compartment Petri dishes (90 x 15 mm). Two 2-day-old *L. sativa* seedlings were placed into one compartment containing MS-A, and the second compartment contained a culture medium such as Nutrient agar (N-A), Methyl Red & Voges Proskauer Agar (MRVP-A) or MS-A. BCT9 suspensions (15 µL) with an initial absorbance (AB) of 0.1, 0.2, 0.5 or 0.7, measured at λ 600 nm, were spread on media. Non-inoculated plates were used as controls. Viable cell counts associated with each AB measurement were determined by performing serial dilutions and obtaining plate counts. The plates were sealed and distributed in a random design. *L. sativa* seedling growth was measured on day 10 (Blom et al., 2011; Velázquez-Becerra et al., 2011).

5.2.5 Growth curve of *Bacillus* sp. BCT9. A growth curve was constructed by tracing cell growth (AB at 600 nm) versus incubation time. Bacteria were inoculated into 250-mL Erlenmeyer flasks containing 50 mL of Nutrient Broth (N-B), Methyl Red & Voges Proskauer Broth (MRVP-B) or Murashige & Skoog Broth (MS-B) and incubated for 48 h

at 28°C with shaking (160 rpm). After incubation, aliquots of each culture medium were transferred into tubes containing a defined culture medium at a 1:10 ratio (inoculum:culture medium). The tubes were incubated at 28°C with shaking (160 rpm), and AB values were measured relative to culture medium alone (n=3) (Campos et al., 2014; Asari et al., 2016).

5.2.6 Microscopic examination of *Bacillus* sp. BCT9. To evaluate BCT9 cell size, a single BCT9 colony grown in MS-A, N-A or MRVP-A was dissolved in sterile distilled water. Cells were photographed using a variable pressure scanning electron microscope with a STEM SU-3500 transmission module (SEM-STEM; Hitachi-Japan).

5.2.7 GC-MS analysis of VOCs emitted by *Bacillus* sp. BCT9. The bacterial isolate was grown in a specific culture medium (MRVP-B) for 24 h before experiments were performed. Twenty-five milliliters of inoculum were added to a 500-mL flask containing 250 mL of culture medium for 19 h at 34°C; during this period, volatiles were collected via solid phase microextraction (SPME) using a PDMS/DVB fiber (Supelco, Inc., Bellafonte, PA, U.S.A). The fiber was conditioned previously at 250°C under helium flow for 10 min. The collected volatiles were desorbed at 250°C for 2 min in the injector compartment of a gas chromatograph (Model Focus, Thermo Electron Corporation, Waltham, USA) coupled to a mass spectrometer (Model DSQ, Thermo Electron Corporation). Chromatographic separation was performed with a DB-1 column (30 m x 0.2 mm x 0.33 µm). Compounds were carried by helium flow (1.0 mL min^{-1}). The GC oven temperature was programmed to ramp from 40 to 250˚C at 5°C/min. Mass spectra were acquired from 35 to 500 a.m.u., and an electronic impact at 70 eV was used for fragmentation with an ion source temperature set at 200°C. Compounds were identified by comparing mass spectra (MS) obtained with those in the NIST library. Additionally, volatile organic compounds were identified by

comparing Kovats indices (KIs) with corresponding commercial standards by injecting an alkane series (C9–C26). Experimental KIs were compared with the theoretical KIs of synthetic standard compounds reported in the Pherobase and NIST databases (NIST ver. 2.0, Thermo). Analyses were performed three times (Parra et al., 2009; Tampe et al., 2016).

5.2.8. Modulation of *L. sativa* growth by bioactive volatile compounds. Bioassays were performed in two-compartment Petri dishes (90 x 15 mm) with two 2-day-old *L. sativa* seedlings in one compartment containing MS-A. In the first stage of the assay, a sterile paper disk (Whatman N^{o}1) was impregnated with 20 µL of a diluent containing 50,000 ppm, 50 ppm or 0.05 ppm of each compound. The ketones 2-nonanone, 2-undecanone, 2-tridecanone and 2-pentadecanone were diluted in hexane. 3-hydroxy-2-butanone and 2,3-butanediol were diluted in water. Each compound was placed on the opposite side of a compartment containing the same medium with respect to the seedlings (Groenhagen et al., 2013). In the second stage of the assay, the tested compounds were mixed with a lanolin solution (0.16 g mL^{-1}), resulting in the slow release of the compound at a 1:1 ratio to achieve the same final concentrations. The compound solutions were applied to a 1-cm section of the central partition. Seedlings that were not exposed to volatile compounds were used as controls (Zou et al., 2010).

5.2.9 Measurement of plant growth. Numbers of lateral roots were recorded ten days after inoculation. Lateral root length, primary root length and shoot length were measured using an electronic Scale Master Pro curvimeter. Dry weight was measured after seedlings were exposed to a temperature of 45°C for 3 days to remove water.

5.2.10 Statistical analyses. Data were analyzed using Statistix v10. Differences in *L. sativa* growth parameters resulting from VOCs treatments were analyzed by performing analysis of variance (ANOVA) and LSD tests ($P \leq 0.05$).

5.3 RESULTS

5.3.1 Growth curve and microscopic description of *Bacillus* sp. BCT9. To describe the growth of *Bacillus* sp. BCT9, growth curves were constructed for the three culture media (Fig. 1). BCT9 cultivated in N-B demonstrated a lag phase from hour 0 to hour 15 and a log phase from hour 15 to 32 h, reaching stationary phase after 32 h (Fig. 1a). BCT9 cultivated in MS-B had a lag phase for the first 10 hours and a log phase between 10 to 72 h, reaching stationary phase after 72 h (Fig. 1a). The MRVP-B medium culture grew more rapidly than the cultures grown in the other media, with a lag phase from 0 to 5 h and a log phase from 5 to 9 h. Bacterial growth remained constant after 9 h (Fig. 1b).Photographs of surfaces measuring 84.5 x 59.7 μm^2 in size revealed fewer numbers of cells when BCT9 was grown in MS-A compared with N-A and MRVP-A, indicating that growth was dependent on the culture medium and not cell size (Fig. 1c-e). Larger cell sizes were observed when BCT9 was grown in MS-A (2.76 μm) compared with the growth in N-A (2.2 μm), while an intermediate cell size was observed in MRVP-A (2.4 μm) (Fig. 1f).

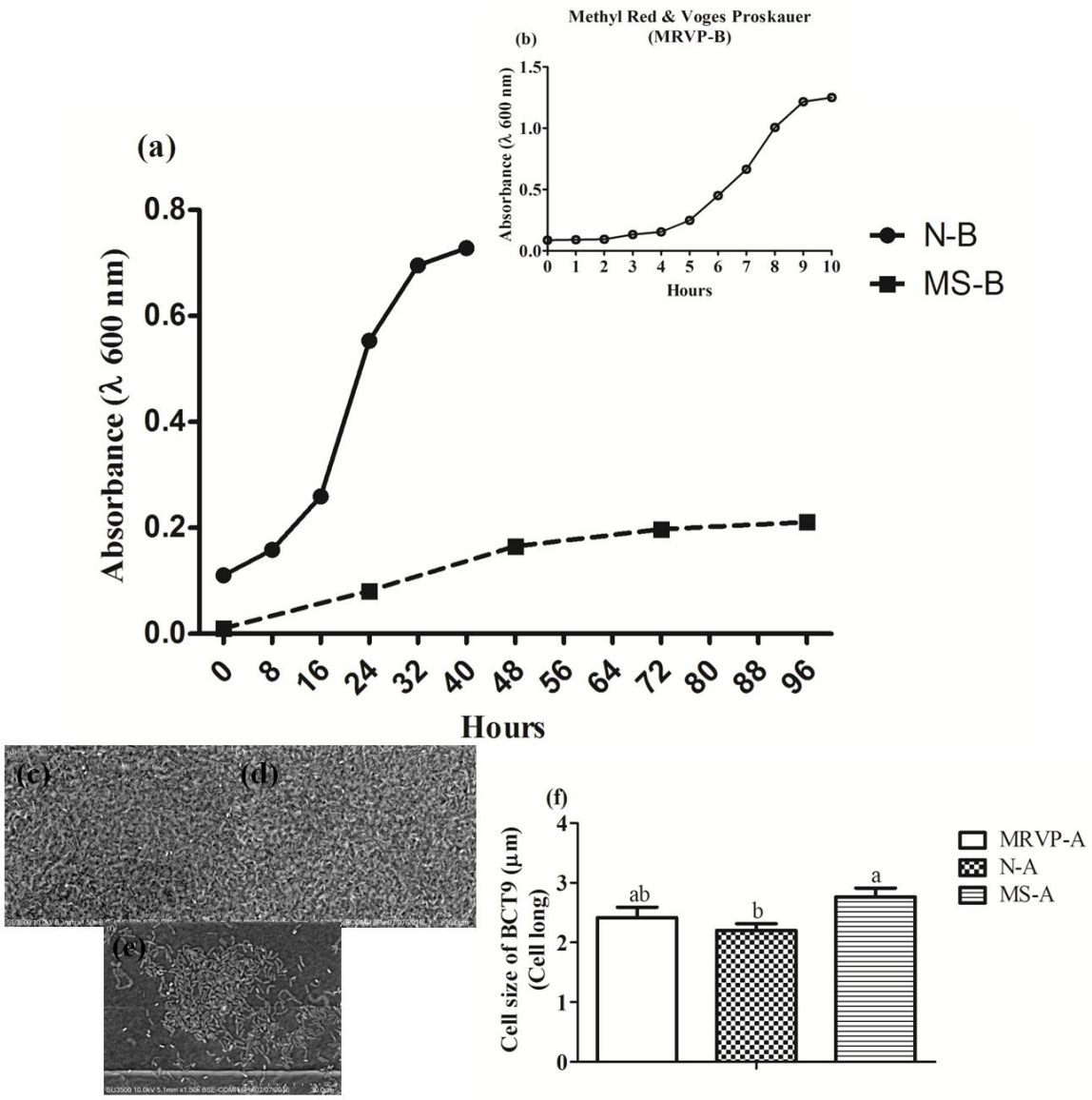


Figure 1. Growth and microscopic description of *Bacillus* sp. BCT9. Growth curve of BCT9 in (a) Nutrient, Murashige & Skoog media and (b) Methyl Red & Voges Proskauer at 28°C and shaking at 160 rpm (each point represents the average of three replicates, and bars indicate standard error. Error bars only appear to be absent because they are covered by the point representing the average). Cell sizes (long cell) of *B. subtilis* BCT9 in the three culture media (g): (d) MRVP-A, (e) N-A and (f) MS-A (n=3).

5.3.2 *L. sativa* growth is modulated by different cell densities of *Bacillus* sp. BCT9. The growth of *L. sativa* seedlings was modulated by different concentrations of BCT9 (Fig. 2 a-e). Significant increases in the number of lateral roots were observed under the following conditions: a) an increase of 490% using MRVP-A medium at AB 0.1 and b) an increase of 221 % using N-A medium at AB 0.1 (Fig. 2a) ($p \leq 0.05$). Additionally, dry weight (Fig. 2b) was significantly increased for all cell densities compared to controls when BCT9 was inoculated in all culture media. Furthermore, increased root length was observed at low densities (AB: 0.1 and 0.2) for cultures grown in N-A, which also achieved the highest average growth (60%) (Fig. 2c). Regarding shoot length, the best yield was obtained for cultures in MRVP-A at AB 0.5 (63%) (Fig. 2d). Finally, the lateral root length was significantly increased when *Bacillus* sp. BCT9 was grown in MRVP-A and N-A media at AB 0.5 (113 and 153%) and AB 0.7 (84 and 103%) (Table 1). In our study, Methyl Red & Voges Proskauer medium was utilized to identify VOCs due to (a) the ability of BCT9 VOCs to induce *L. sativa* growth and (b) the enhanced ability of BCT9 to grow in this medium. Therefore, this medium was selected to perform the following experiments during this research.

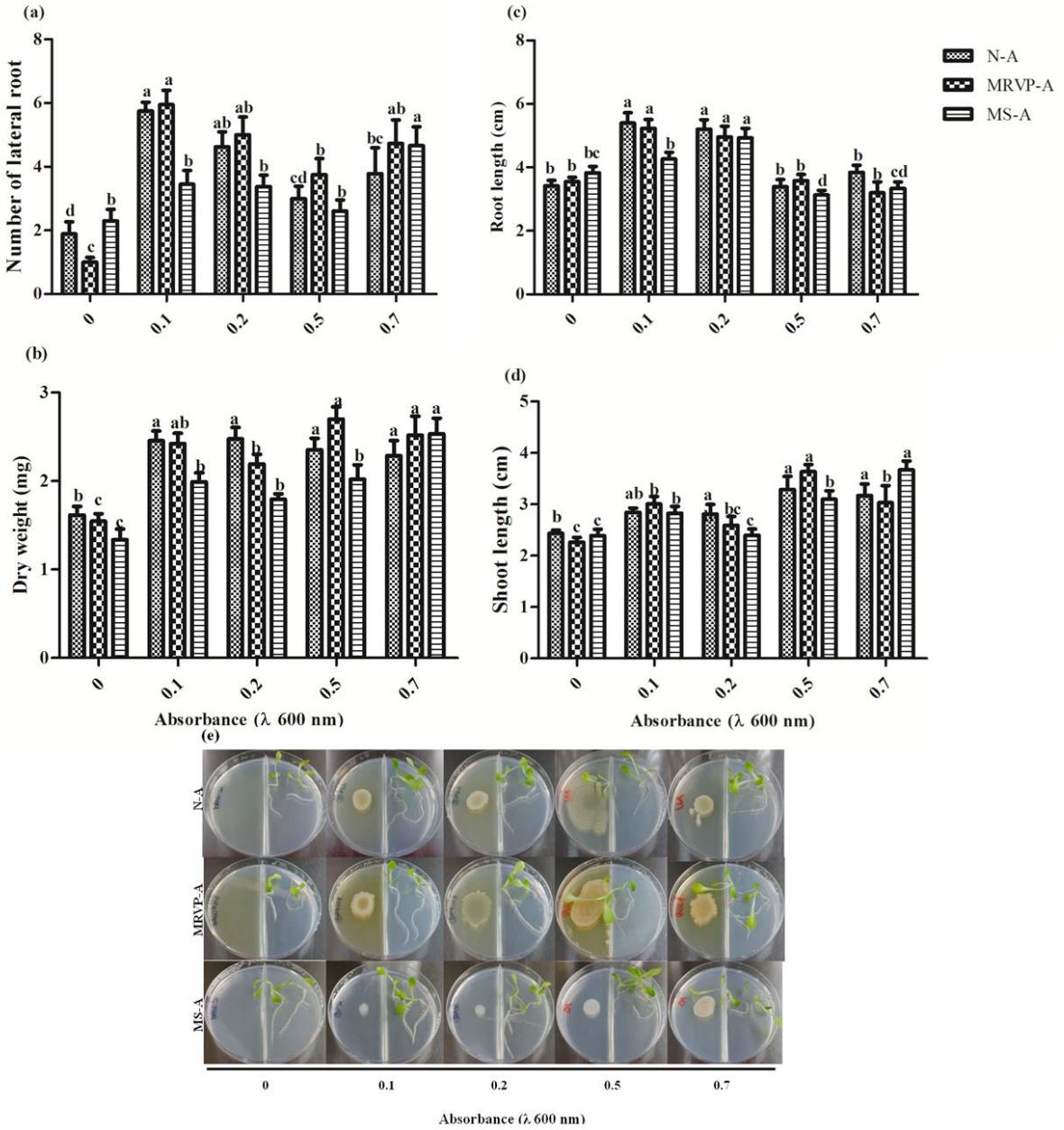


Figure 2. Growth of *L. sativa* seedlings modulated by volatile organic compounds emitted by different bacterial cell densities on day 10. *L. sativa* seedlings were grown in divided Petri plates on Murashige & Skoog medium. The opposite compartment contained nutrient agar (N-A), Methyl Red & Voges Proskauer agar (MRVP-A) and Murashige & Skoog agar (MS-A) inoculated with 15 µL of *Bacillus* sp. BCT9 at an absorbance of 0.1 ($5.0x10^7$ CFU mL^{-1}), 0.2 ($6.0x10^7$ CFU mL^{-1}), 0.5 ($2.7x10^8$ CFU mL^{-1}), or 0.7 ($2.0x10^8$ CFU mL^{-1}); an absorbance of 0 was considered a control (without inoculum) (a-e). The (a) number of lateral roots, (b) dry weight, (c) root length and (d) shoot length were measured on day 10. Letters indicate statistically significant differences as determined by LSD test for each culture medium ($p<0.05$). Bars represent standard error values (n=15-20).

Table 1. Modulation of *L. sativa* seedling lateral root length by VOCs emitted by different cell densities of BCT9 on day 10. BCT9 concentrations were measured as absorbances (AB) of 0.1 ($5.0x10^7$ CFU mL^{-1}), 0.2 ($6.0x10^7$ CFU mL^{-1}), 0.5 ($2.7x10^8$ CFU mL^{-1}) or 0.7 ($2.0x10^8$ CFU mL^{-1}) and were applied in the indicated culture medium. Plates lacking inoculant were considered controls (C). Letters indicate statistically significant differences as determined by LSD test for each column ($p < 0.05$).

Concentration (AB) (λ 600 nm)	**Lateral root length (cm)**		
	Nutrient (N-A)	Murashige & Skoog (MS-A)	Methyl Red Voges Proskauer (MRVP-A)
0.1	0.42 [b]	0.47 [ab]	0.45 [b]
0.2	0.41 [b]	0.34 [b]	0.39 [bc]
0.5	0.76 [a]	0.60 [a]	0.64 [a]
0.7	0.65 [a]	0.51 [a]	0.59 [a]
C	0.32 [b]	0.46 [ab]	0.30 [c]

5.2.3 Identification of volatile compounds emitted by *Bacillus* sp. BCT9. The volatile profile released by BCT9 following growth in Methyl Red & Voges Proskauer medium is shown in Table 2. The largest proportion of released VOCs corresponded to 2-undecanone (52%). Other identified compounds included 3-hydroxy-2-butanone, 2,3-butanediol, 2-nonanone, 2-tridecanone and 2-pentadecanone.

Table 2. Chemical composition of volatile organic compounds released by *Bacillus* sp. BCT9 grown in Methyl Red & Voges Proskauer medium. (RT= retention time; KI= Kovats index; KI exp.= Kovats index experimental; KI lib.= Kovats index database; MS= mass spectra; Std= standard).

Pathway product	RT	Compound	KI exp.	KI lib.		Area (%)	Identification
				NIST	Pheromone		
Pyruvate fermentation	3.9	3-Hydroxy-2-butanone	748	743	739	0.85	MS, Std injection
	5.1	2,3-Butanediol	784	790	779	1.73	MS, Std injection
Fatty acid cycle	13.0	2-Nonanone	1074	1076	1072	3.23	MS, Std injection
	18.7	2-Undecanone	1275	1275	1273	52.77	MS, Std injection
	23.9	2-Tridecanone	1476	1476	1475	23.98	MS, Std injection
	28.6	2-Pentadecanone	1678	1678	1676	17.44	MS, Std injection

5.2.4 The effects of bioactive compounds on *L. sativa* growth. *L. sativa* seedlings exposed to 50 ppm of 2-nonanone mixed with lanolin elicited an increase in root length (22%). Root length also increased following exposure to 3-hydroxy-2-butanone (50 ppm) without lanolin (~9%) and 2-tridecanone (~9-11%) independent of lanolin application. Additionally, shoot length was significantly increased by 31% with low doses of 2-nonanone (0.05 ppm) accompanied by lanolin application. Furthermore, increased dry weight was elicited by 2-tridecanone (0.05 ppm) and 2-undecanone without lanolin (0.05 ppm), resulting in increases of 23% and 25%, respectively. In addition, the application of 2-nonanone without lanolin resulted in increased dry weight (48%) (Table 3) (Fig. 3a).

Finally, lateral root length increased after exposure to 2-nonanone (50 ppm) and 2-undecanone (0.05 ppm) with lanolin, reaching values 62% and 67% higher with respect to controls (Table 4). Interestingly, the exposure of seedlings to high concentrations of methyl-ketones modulated foliar development based on carbon number (Fig. 3b).

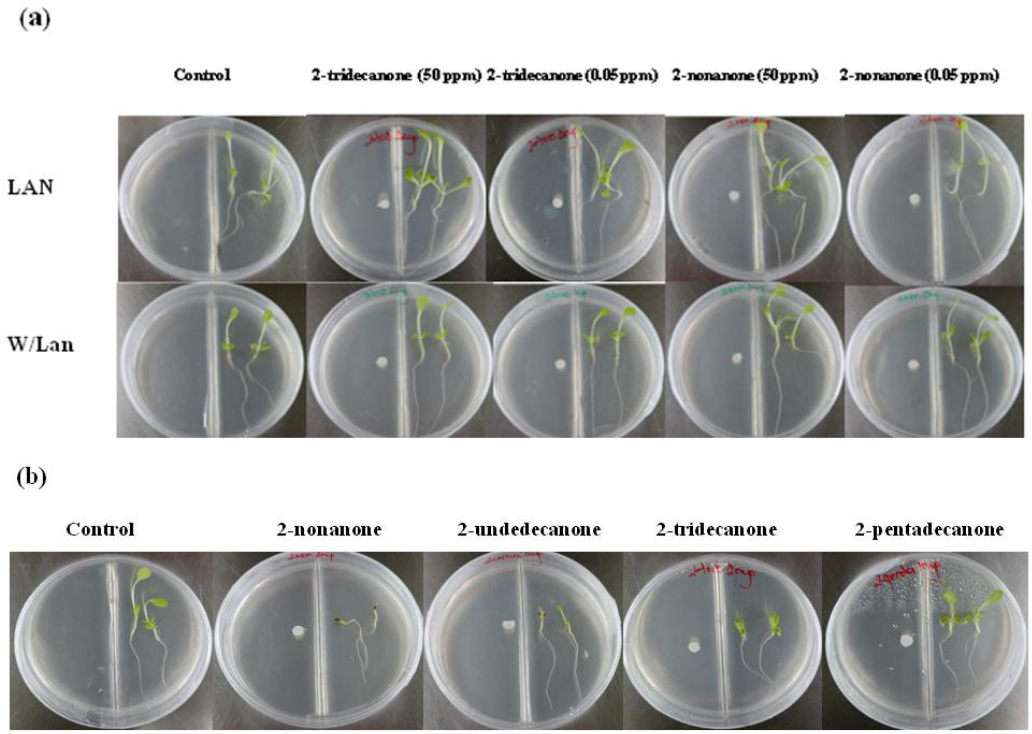


Figure 3. Growth of *L. sativa* seedlings modulated by bioactive volatiles on day 10. *L. sativa* seedlings were grown in divided Petri plates on Murashige & Skoog medium. The images show (a) 2-nonanone and 2-tridecanone as growth inducers at 50 and 0.05 ppm and (b) the modulatory effects of methyl-ketones at the foliar level following exposure to 50,000 ppm. Non-exposed seedlings were considered controls.

Table 3. Increased root length, shoot length and dry weight in *L. sativa* seedlings elicited by 0.05 ppm, 50 ppm or 50.000 ppm of bioactive volatile organic compounds released by *Bacillus* sp. BCT9 after 10 days of exposure. Letters indicate significant differences for each column as determined by LSD test ($p < 0.05$; n= 18-20) (Lan= with lanolin; W/Lan= without lanolin; C=control).

Compound	**Doses (ppm)**	**Root length (cm)**		**Shoot length (cm)**		**Dry weight (mg)**	
		W/Lan	Lan	W/Lan	Lan	W/Lan	Lan
2-Nonanone	50,000	3.05 ^{e}	3.36 ^{h}	0.00 ^{j}	0.00 ^{i}	0.27 ^{f}	0.27 ^{e}
	50	4.86 ^{abc}	5.68 ^{a}	3.52 ^{abcd}	4.52 ^{a}	1.04 ^{bc}	1.42 ^{ab}
	0.05	4.77 ^{abcd}	5.22 ^{bc}	3.21 ^{defg}	4.56 ^{a}	1.03 ^{bc}	1.28 ^{bc}
2-Undecanone	50,000	4.67 ^{abcd}	4.53 ^{fg}	2.25 ^{i}	2.15 ^{g}	0.63 ^{de}	0.49 ^{e}
	50	4.77 ^{abcd}	4.93 ^{bcde}	3.58 ^{abc}	4.10 ^{bc}	1.00 ^{bc}	1.13 ^{cd}
	0.05	4.71 ^{abcd}	4.88 ^{cde}	3.71 ^{a}	4.32 ^{ab}	1.28 ^{a}	1.24 ^{bc}
2-Tridecanone	50,000	4.52 ^{cd}	4.61 ^{ef}	1.99 ^{i}	1.36 ^{h}	0.85 ^{cd}	0.35 ^{e}
	50	4.97 ^{a}	5.16 ^{bc}	3.64 ^{ab}	4.44 ^{a}	1.17 ^{ab}	1.26 ^{bc}
	0.05	4.70 ^{abcd}	5.23 ^{b}	3.38 ^{bcdef}	4.08 ^{bc}	1.31 ^{a}	1.15 ^{cd}
2-Pentadecanone	50,000	4.68 ^{abcd}	5.03 ^{bc}	3.02 ^{g}	3.47 ^{f}	1.25 ^{ab}	1.07 ^{cd}
	50	4.72 ^{abcd}	4.96 ^{bcd}	3.50 ^{abcd}	4.07 ^{bc}	1.05 ^{abc}	0.90 ^{d}
	0.05	4.82 ^{abcd}	4.66 ^{def}	3.55 ^{abc}	3.89 ^{cd}	1.04 ^{bc}	0.91 ^{d}
3-Hydroxy-2-butanone	50,000	4.47 ^{d}	4.94 ^{bcde}	3.00 ^{g}	3.77 ^{de}	0.44 ^{ef}	0.98 ^{d}
	50	4.96 ^{a}	4.92 ^{bcde}	3.10 ^{fg}	4.06 ^{bc}	0.63 ^{de}	1.06 ^{cd}
	0.05	4.90 ^{ab}	5.18 ^{bc}	3.48 ^{abcde}	3.89 ^{cd}	0.55 ^{de}	1.06 ^{cd}
2,3-Butanediol	50,000	4.67 ^{abcd}	4.22 ^{g}	2.64 ^{h}	3.48 ^{f}	0.49 ^{ef}	1.64 ^{a}
	50	4.71 ^{abcd}	4.94 ^{bcde}	3.17 ^{efg}	3.54 ^{ef}	0.93 ^{bc}	1.47 ^{ab}
	0.05	4.62 ^{abcd}	4.89 ^{bcde}	3.27 ^{cdefg}	3.54 ^{ef}	1.10 ^{abc}	1.45 ^{ab}
C		4.56 ^{bcd}	4.65 ^{def}	3.13 ^{fg}	3.48 ^{ef}	1.04 ^{bc}	0.96 ^{d}

Table 4. Increased lateral root length and number of lateral roots of *L. sativa* seedlings elicited by 0.05 ppm, 50 ppm or 50.000 ppm of bioactive volatile organic compounds released by *Bacillus* sp. BCT9 after 10 days of exposure. Letters indicate significant differences for each column as determined by LSD test ($p < 0.05$; n= 18-20) (Lan= with lanolin; W/Lan= without lanolin; C= control).

Compound	**Doses (ppm)**	**Lateral root length (cm)**		**Number of lateral root**	
		W/Lan	Lan	W/Lan	Lan
2-Nonanone	50,000	*	*	0.00 ^{g}	0.00 ^{f}
	50	0.62 ^{abcde}	0.60 ^{a}	0.70 ^{abcd}	0.72 ^{ab}
	0.05	0.68 ^{abc}	0.43 ^{abcd}	0.40 ^{bcdefg}	0.45 ^{bcde}
2-Undecanone	50,000	*	0.53 ^{ab}	0.00 ^{g}	0.05 ^{ef}
	50	0.75 ^{a}	0.41^{abcd}	0.44 ^{bcdef}	0.38 ^{bcdef}
	0.05	0.42 ^{cde}	0.62 ^{a}	0.55 ^{bcde}	0.50 ^{bcd}
2-Tridecanone	50,000	0.26 ^{e}	0.45 ^{abcd}	0.10 ^{fg}	0.22 ^{def}
	50	0.32 ^{e}	0.46 ^{abcd}	0.40 ^{bcdefg}	0.40 ^{bcdef}
	0.05	0.49 ^{bcde}	0.45 ^{abcd}	0.75 ^{abc}	0.50 ^{bcd}
2-Pentadecanone	50,000	0.78 ^{a}	0.50 ^{abc}	0.27 ^{defg}	0.30 ^{cdef}
	50	0.48 ^{bcde}	0.43 ^{abcd}	0.30 ^{defg}	0.40 ^{bcdef}
	0.05	0.64 ^{abcde}	0.52 ^{ab}	0.45 ^{bcdef}	0.25 ^{def}
3-Hydroxy-2-butanone	50,000	0.57 ^{abcde}	0.31 ^{cde}	0.36 ^{cdefg}	0.44 ^{bcde}
	50	0.69 ^{ab}	0.38 ^{bcd}	0.45 ^{bcdef}	0.15 ^{def}
	0.05	0.56 ^{bcde}	0.1 ^{e}	0.22 ^{efg}	0.10 ^{def}
2,3-Butanediol	50,000	0.50 ^{bcde}	0.36 ^{bcde}	0.70 ^{abcd}	0.22 ^{def}
	50	0.75 ^{a}	0.52 ^{ab}	1.00 ^{a}	0.50 ^{bcd}
	0.05	0.71^{ab}	0.38 ^{bcd}	0.70 ^{abcd}	0.71 ^{abc}
C		0.60 ^{abcde}	0.37 ^{bcd}	0.82 ^{ab}	0.94 ^{a}

5.4 DISCUSION

According to previous studies, certain bacterial strains release VOCs that act as chemical messengers with the ability to induce the growth of *A. thaliana* (Bailly and Weisskopf, 2012; Chung et al., 2015; Kai et al., 2016). Additionally, factors such as the culture medium and bacterial concentration are critical parameters that promote growth; however, little is known about their effects on horticultural species, of which *L. sativa* has emerged as a model to assess VOC application.

Based on our data, VOCs released by *Bacillus* sp. BCT9 induced *L. sativa* growth depending on the cell density applied, independent of the culture medium. The differential effects of various cell densities on growth parameters may be attributable to the initial cell density or viable cell numbers of BCT9 inoculated in the culture medium; for example, the application of $5.0x10^{7}$ CFU mL^{-1} (AB 0.1) elicited root growth, while $2.7x10^{8}$ CFU mL^{-1} (AB 0.5) induced an increase in shoot length. Another potentially critical factor is the differential growth rate of *Bacillus* sp. BCT9 in the three culture media tested due to their composition. According to the generated by growth curves, more rapid growth was observed in MRVP-B compared to MS-B, with an intermediate response in N-B. These effects are attributable to the medium composition: MRVP-B contained D(+) glucose, peptone and phosphate buffer, of which glucose is a relevant sugar source for *Bacillus* growth according to a report by Bailly and Weisskopf (2012), while N-B contained beef extract and peptone and lacked a direct sugar source. In contrast, MS-B is a mineral medium that contains saccharose as a carbon source, which must be hydrolyzed into monomers for use. The observed effects suggest the ability of bioactive volatiles to modulate *L. sativa* growth depending on bacterial cell density, supporting the critical nature

of this parameter when testing the ability of a bacterial strain to elicit plant growth. Furthermore, based on the cell size (long cell) of *Bacillus* sp. BCT9, both the culture medium and strain surface appear to be relevant factors affecting the qualitative and quantitative characteristics of VOCs released by this strain.

Methyl-ketones (2-nonanone, 2-undecanone, 2-tridecanone, 2-pentadecanone), 3-hydroxy-2-butanone and 2,3-butanediol were released when BCT9 was grown in Methyl Red & Voges Proskauer medium. Methyl-ketones are derived from the fatty acid pathway, which typically utilizes acetyl-CoA as a substrate and reacts with malonate to form fatty acids, while 3-hydroxy-2-butanone and 2,3-butanediol are derived from pyruvate fermentation (Schulz and Dickschat, 2007; Audrain et al., 2015).

According to our results, methyl-ketones applied at high concentration significantly modulate *L. sativa* growth at the leaf level. Notably, this effect has not previously been reported. Furthermore, 3-hydroxy-2-butanone, 2,3-butanediol and certain ketone compounds induced increases in root length, shoot length or dry weight at low concentrations, in contrast to the actions of 2-nonanone and 2-undecanone, which increased the lateral root length only at 50 and 0.05 ppm without lanolin. Thus, low doses demonstrated higher growth-inducing capacity in accordance with the reported results for other volatiles. Previously, Ryu et al. (2003) described the ability of 2,3-butanediol to induce the growth of the *A. thaliana* leaf surface. Diverse studies support the results obtained in this work. For example, Zou et al. (2010) observed an in increase in the fresh weight of *A. thaliana* following the application of 0.5 μg/μL 2-pentylfuran for 15 days, while Blom et al. (2011) reported an increase in the same parameter with 1 μg/μL indole following 21 days of exposure. Groenhagen et al. (2013) also revealed the ability of

Dimethyl disulfide and Acetophenone, both at a concentration of 1 μg/μL, to increase biomass in the same plant species after 3 weeks, and Ann et al. (2013) noted the increased fresh weight of tobacco following 1 week of exposure to 1 and 10 ppm of 3-hydroxy-2-butanone. A recent study by Bhattacharyya et al. (2015) indicated that indole induced increases in shoot length, primary root length, lateral root number and fresh weight at concentrations ranging from 0.01 μg/μL to 0.250 μg/μL; furthermore, according to Park et al. (2015), 13-tetradecadien-1-ol, 2-methyl-η-1-tridecene and 2-butanone at 50 and 5 ng increased the fresh weight of tobacco after 4 weeks.

The evaluation of bioactive compounds released by bacteria during the early stages of plant growth allows us to determine if such compounds exert toxic effects on target plants through a series of physiological changes that are susceptible to the actions of synthetic compounds. Older seedlings or plants exhibit greater resistance to these stimuli due to increased mechanical protection conferred primarily through cuticle development (Fernandez et al., 2016). In addition, evaluation of the effects of volatile organic compounds permits the detection of modulators that induce growth or development, contributing to seedling quality. In summary, volatiles promote increased crop productivity by enhancing the survival of seedlings through improved root development.

Physiologically, the modulatory effects of VOCs on *L. sativa* growth may be attributable to their ability to increase or decrease cellular signals associated with physiological processes involved in phytohormone synthesis or regulation (Ryu et al., 2003; Zhang et al., 2008; Minerdi et al., 2011). According to Zhang et al. (2007, 2008, and 2009), VOCs emitted by *B. subtilis* GB03 exert diverse effects on *A. thaliana* at the cellular and molecular levels. However, future studies must elucidate the mechanisms associated

with the specific effects of VOCs on *L. sativa*. To our knowledge, this study implemented under "*in vitro*" conditions over a period of 10 days provides a relevant evidence of the effects of culture conditions as critical parameters for bacterial volatile production to induce the growth of *L. sativa*. Additionally, this is the first study to expose a leaf vegetable to methyl-ketone chains and evaluate dose effects, suggesting VOCs may be applied as a sustainable biotechnology tool for horticulture as an alternative or complementary strategy to reduce the application of chemical products. In conclusion, volatile organic compounds released by BCT9 increase *L. sativa* growth in a concentration (cell density)-dependent manner; specifically, the greater cell densities increase shoot length and lateral root length, while low cell densities stimulate root length. Furthermore, all cell densities induce increases in dry weight. Finally, ketone compounds induce *L. sativa* root and shoot growth at low concentrations (0.05 and 50 ppm).

CHAPTER VI:

Physiological response of *Lactuca sativa* exposed to 2-nonanone

6.1 INTRODUCTION

In the last years, volatile organic compounds (VOCs) acting as plant growth inducers have emerged as a new complementary or alternative tool to the fertilizers, because its capacity to act as signal molecule modulating growth and nutrition of plants with lower cost compared to genetically modified plant and strategies based on chemical fertilization (Bitas et al., 2013; Kanchiswamy et al., 2015; Kai et al., 2016). VOCs are characterized by having low molecular weight (<300 g mol^{-1}), high vapor pressure (0.01 kPa at 20°C) and ability to diffuse through soil, air and water (Audrain et al., 2015). Furthermore, these compounds may belong to different chemical classes as alcohols, acids, ketones, terpenes, hydrocarbons, nitrogen and sulfur containing compounds (Schulz and Dickschat, 2007; Korpi et al., 2009).

On the other hand, bioactive VOCs can be incorporated into a polymeric matrix with the aim of controlling the release of the specific compound. In this sense, lanolin is a wax matrix used to apply VOC due to minimizes the initial burst into the "bioassay atmosphere", extending seedling exposition to VOC tested (Kessler and Baldwin, 2001; Ryu et al., 2003; Groenhagen et al. 2013). The first study was performed by Ryu et al. (2003), which showed that 2,3-Butanediol released by *Bacillus subtilis* GB03 induced the increase of surface leaf area on *Arabidopsis thaliana*. Later, Zou et al. (2010) reported that 2-pentylfuran emitted by *B. megaterium* XTBG-34 elicited the increase of fresh weight in the same plant species. Furthermore, a study conducted by Velázquez et al. (2011) showed that dimethylhexadecylamine emitted by *Arthrobacter agilis* UMCV2 increased fresh weight, stem length, root length and lateral root density on *Medicago sativa*. Subsequently, Ann et al. (2013) reported that 3-hydroxy-2-butanone induced the increase of fresh weight

on tobacco. Recently, Fincheira et al. (2016a,b) reported that VOCs released by *Bacillus* sp. BCT9, such as 3-hydroxy-2-butanone, 2,3-butanediol, 2-nonanone, 2-undecanone, 2-tridecanone and 2-pentadecanone, showed their ability to increase shoot length, root length and dry weight in *Lactuca sativa* seedlings at low concentrations (50 and 0.05 ppm), and lateral root length was increase when they were exposed to 2-nonanone at 50 ppm (Fincheira et al. 2016b).

It highlights that "seedlings" are used to test VOCs as growth inducers due to physiological changes are detected after short exposure in this stage. Studies in *A. thaliana* have showed that genetic and proteomic changes are induced by VOCs released from *B. subtilis* GB03. The study performed by Zhang et al. (2007) reported that genes related to metabolism, growth, stress and cellular signaling are modulated by volatiles. Furthermore, Kwon et al. (2010) showed that proteins belong to subcelullar location, molecular function and biological processes were regulated after VOCs exposition. In addition, some compounds are associated with physiological response. For instance, Ryu et al. (2003) reported that 2,3-butanediol increased leaf surface area through cytokinin pathway and Bhattacharyya et al. (2015) indicated that Indole elicited plant growth through cytokinin, auxin and brassinosteroid pathways.

Based on the above mentioned, it has been suggested that bioactive volatiles can be used as growth inducers in horticultural species as novel strategy to the synthetic fertilizer due to its ability to acts as signal molecule that trigger physiological effects for inducing growth in target seedlings without physical contact, which has been shown in previous studies (Blom et al., 2011; Park et al., 2015; Asari et al., 2016). Considering that there is scarce information associating action mechanism or physiological effects with a specific

VOC, *Lactuca sativa* emerge as a model to evaluate bioactive VOCs on leaf vegetable physiology. Hence, in this study 2-nonanone was used as a signal molecule (Fincheira et al., 2016b) to prospect physiological effects on *L. sativa*, which is considered as inexpensive, simple, reliable and sensitive species to environmental stress and it is used widely to evaluate phytotoxicity effects of compounds (Valerio et al., 2007; Charles et al., 2011). Based on the above, this study was focused on the evaluation of morphological and physiological effects on *L. sativa* seedlings exposed to 2-nonanone through techniques that allow the image acquisition "*In vivo*" with high resolution to visualize cellular structures for understanding plant response.

6.2 MATERIALS AND METHODS

6.2.1. Plant material and growth conditions. *L. sativa* seedlings of commercial origin (Green lettuce cv Reina de mayo asepo, semillas Fito, S.A) were employed to carry out the experiment. The seeds surface were sterilized for 8 min with 3% sodium hypochlorite and washed 8 times with sterile distilled water. Later, seeds were germinated on the surface of Petri dishes with Murashige and Skoog basal medium with vitamins 0.5X (PhytoTechnology Laboratories, LLC™), containing 0.8% agar and 1.5% sucrose (MS-A). Then, Petri dishes were placed into a conditioned room with 16/8-h light-dark cycle under 36-W fluorescent light. The temperature was maintained between 20-25°C. Finally, germinated seedlings were transferred into two-compartment Petri dish after 2 days for experimental uses (Minerdi et al., 2011).

6.2.2 *L. sativa* growth induced by 2-nonanone. Two 2-day-old *L. sativa* seedlings were placed into a two-compartment Petri dish (90 x 15 mm) containing MS-A prepared according to described in the previous point. In the second compartment, containing the same culture medium, a sterile paper disk (Whatman N^o1) impregnate with 2-nonanone was placed. Two methodologies were employed to apply the ketone: T_1 = 20 µL of 2-nonanone diluted directly in hexane and T_2= 20 µL of 2-nonanone + lanolin solution (0.16 g mL^{-1}) (ratio 1:1) with the same final concentration. 2-nonanone solution was applied to 1 cm of the central division of the Petri dish. Non-exposed seedlings to 2-nonanone were used as a control. Seedlings were exposed to 2-nonanone during 10 days (Zou et al., 2010; Groenhagen et al., 2013). The numbers of lateral roots were measured immediately. Furthermore, lateral root length, primary root length and shoot length were measured through electronic curvimeter Scale Master Pro.

6.2.3 Analysis of *L. sativa* morphology. Three seedlings of *L. sativa* exposed to T_1 and T_2 for 10 days were chosen randomLy to evaluate their morphology. The evaluations of surface epidermal cells of leaf seedlings were performed in the central zone. Besides, root tips (1 cm) were excised to perform microscopically analyses. The ImageJ software was used to perform length measurements (Tajima and Kato, 2013).

6.2.3.1 Scanning electron microscopy. Root and leaf tissue were mounted on carbon stub to analyze in a variable pressure scanning electron microscope with transmission module (VP-SEM SU 3500 Hitachi-Japan). The images were captured with backscattered electron detector (BSE) with 10.5 mm (leaf) or 5 mm (root) of working distance (WD). The beam energy used was 10.5 KeV (leaf) or 5 KeV (root). The samples were observed using

reflected electrons at 6 Pa (Angeles et al., 2004). The root hair lengths were expressed in μm and root hair density by number of root hairs per 100 μm.

6.2.3.2 Scanning electron microscopy coupled to X-ray elemental microanalysis. Three different zones were analyzed at leaf and root level on *L. sativa* seedlings to evaluate distribution and intensity of elements presents in the surface of tissues. The samples were mounted on carbon stub. The analyses were performed by Scanning Electron Microscopy (SEM) with Energy-dispersive X-ray spectroscopy detector (EDX). BSE and EDX spectra were recording using QUANTAX 100 (Bruker-Germany). The beam energy was set at 15 KeV. The samples were observed using reflected electrons at about 6 Pa to visualize the outlines of cells. The analyses were performed in three different zones for each sample to evaluate elements distribution. The elements were evaluated according to intensity and percentage presence.

6.2.3.3 Confocal laser microscopy. Tissue analyzes were performed by Confocal laser microscopy (CLSM FV 1000 Olympus-Japan). Root samples were mounted on microscopic slides and observed directly without staining through Nomarski image to visualize root epidermal cell morphology. Besides, leaf samples were incubated for 30 minutes with 0.1% safranin at 4°C under darkness conditions. Latter, leaf samples were washed 4 times with sterile distilled water (Angeles et al., 2004; Bond et al., 2008). To visualize leaf morphology, the samples were analyzed by fluorescence emission of safranin to evaluate changes in the cell wall through λ excitation/emission 546/590 nm (green color). In addition, the autofluorescence of the tissue was evaluated at 488/530 nm and 633 / 650-750 nm, which are characteristic of chlorophyll (red color) (Kodama, 2016). The

images were processed with software FV10 v 0.2c. The sizes of epidermal cells and length of stomata aperture were expressed in μm.

6.2.4 Statistical analysis. The results obtained after treatments applications were analyzed by Statistix v10 using T-Student test and analysis of variance (ANOVA) and LSD test ($P \leq 0.05$).

6.3 RESULTS

6.3.1. Growth induction on *L. sativa* elicited by 2-nonanone. Numbers of lateral roots were not affected by the compound presence (Fig. 1a). On the contrary, 2-nonanone alone and incorporated into lanolin (T_1 and T_2) increased root length growth in 16.5 and 18.1% respectively (Fig. 1b). Shoot length was significantly incremented when plants were exposed to both treatments in 11.0 and 18.2% respectively (Fig. 1c). Lateral root length was significantly incremented in 76.3 % when plants were exposed to 2-nonanone mixed with lanolin (Fig. 1d).

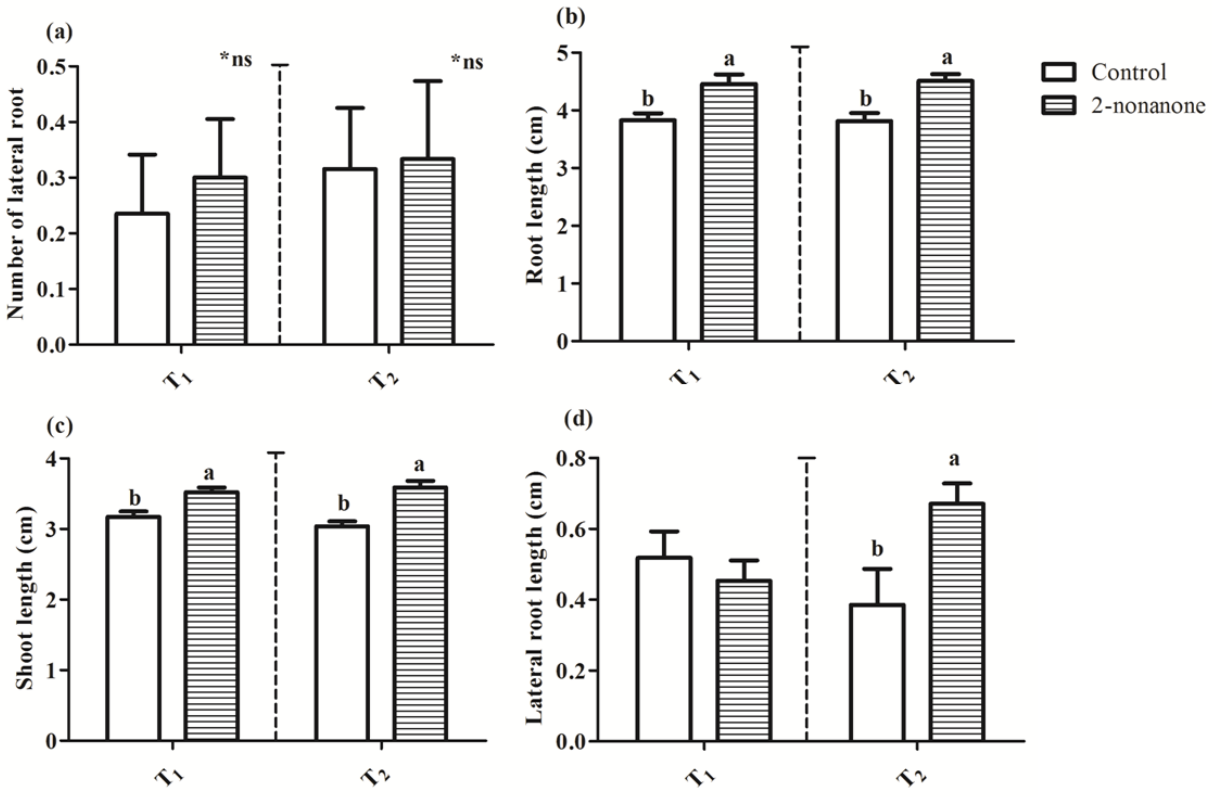


Figure 1. Effects of 2-nonanone on *L. sativa* growth. Seedlings were placed on Murashige & Skoog medium and exposed to two delivey volatile systems: T_1: 2-nonanone (abrupt) and T_2: 2-nonanone + Lanolin (controlled), both with 50 ppm of bioactive compound. Plant growth parameters evaluated were (a) number of lateral root, (b) root length, (c) shoot length and (d) lateral root length. Error bars indicate error standard of mean (N=20). The letters indicate differences statistically significant according to T-Student test ($p < 0.05$) performed for each delivery system (*ns= no significant difference).

6.3.2. Description of *L. sativa* root. To evaluate root development and external morphology of *L. sativa* a scanning electron microscopy technique was performed to visualize the tissue. The results indicated that 2-nonanone modified the root development (Fig 2 a-c). Furthermore, seedlings exposed to T_1 exhibited a 106.8% increase in root hair density and a significant increase of numbers of root hair was observed (6.1 versus 2.9 of the control) (Fig. 2d). Root hair length increased 105.5 % in seedlings exposed to T_1 respect to control, 421.7 and 205.2 μm respectively (Fig. 2e). The size of surface epidermal cells

did not differ after 2-nonanone exposure, independently of treatment applied with average values ranging from 47.3 to 54.5 µm (Fig. 3a). In addition, cell morphology or structures were not altered after 2-nonanone exposition, suggesting that the VOC does not harm on tissue (Fig. 3b-d).

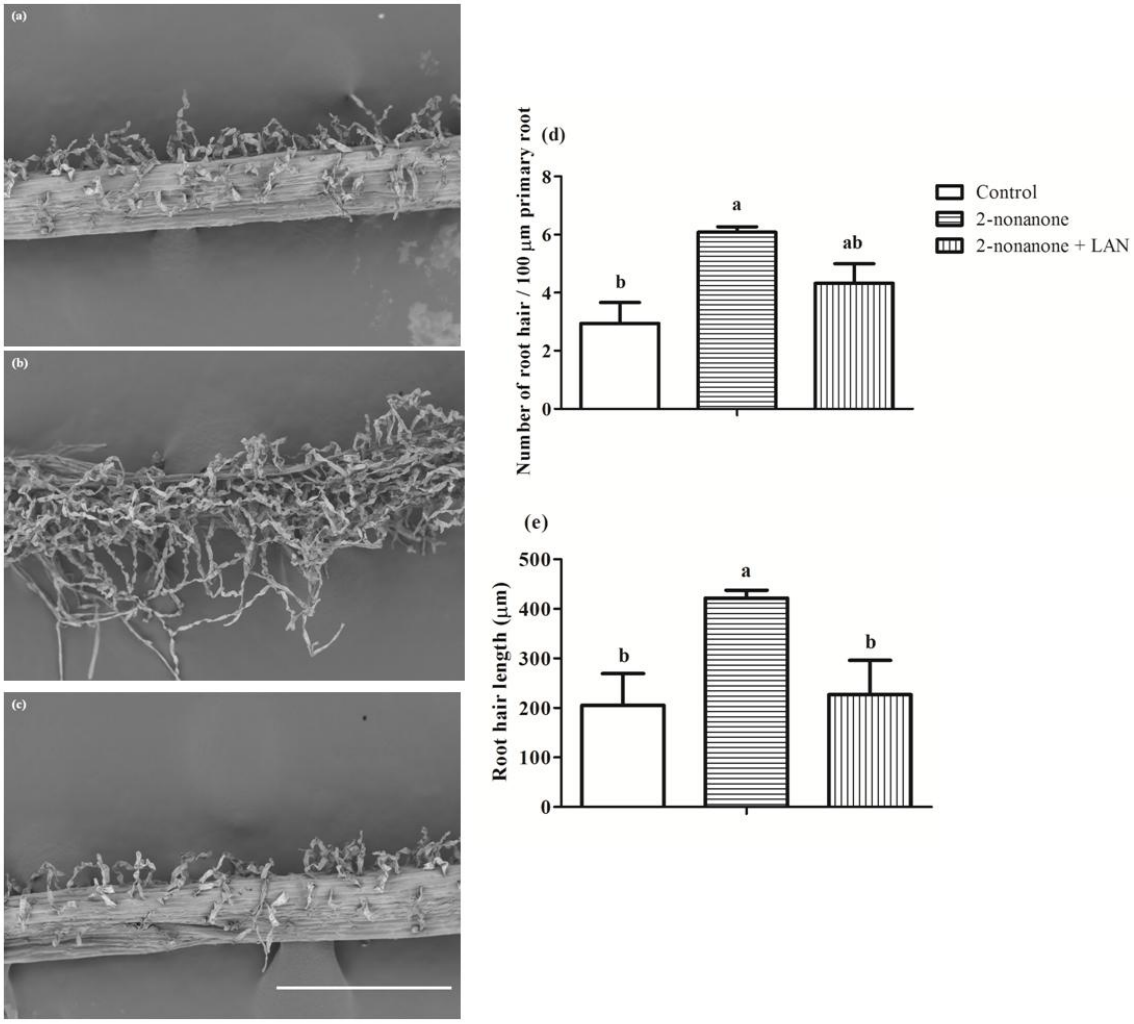


Figure 2. Representative photographs of root hair development of *L. sativa* after 10 days of exposure to 2-nonanone (50 ppm). The *L. sativa* seedlings were placed in the surface of Murashige & Skoog medium. (a) control, (b) T_1: 2-nonanone (abrupt system) and (c) T_2: 2-nonanone + Lanolin (controlled system). Parameters evaluated were (d) root hair density and (e) root hair length. Error bars indicate error standard of mean (N=3). The letters indicate differences statistically significant according to ANOVA (LSD test, $p < 0.05$). Scalebars: 400 µm.

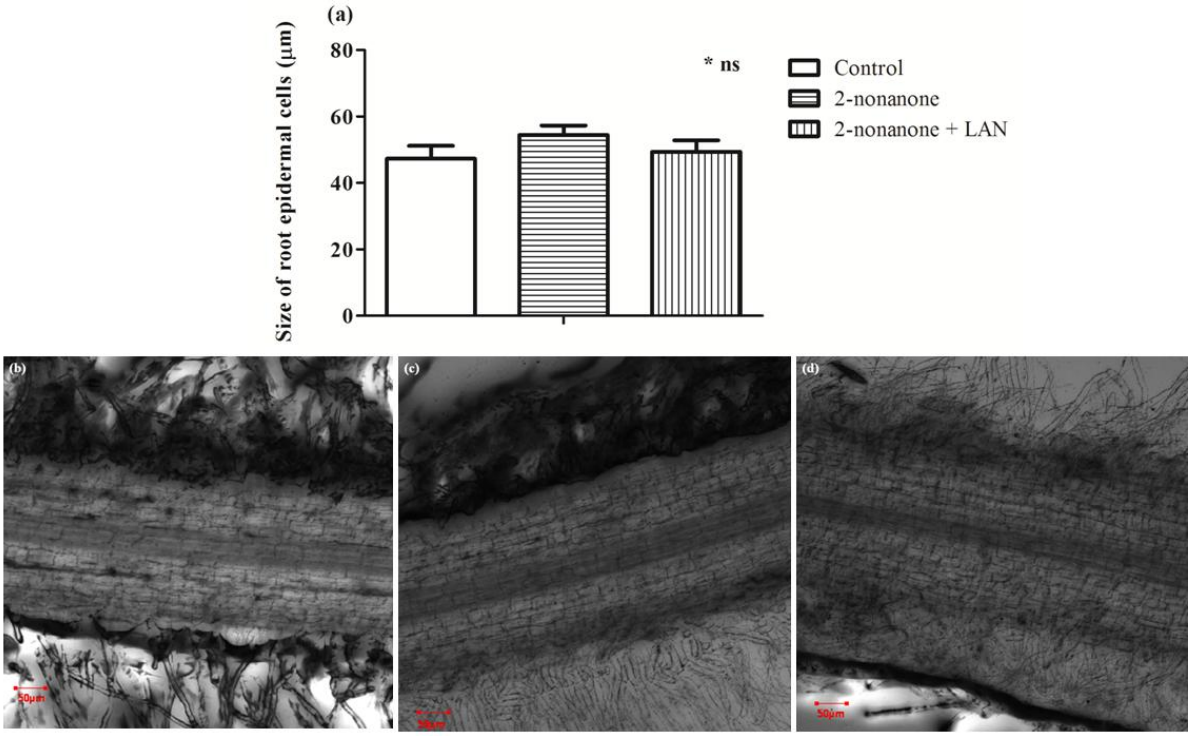


Figure 3. Representative photographs of size of root epidermal cells and morphology of *L. sativa* seedlings exposed to 2-nonanone (50 ppm). *L. sativa* seedlings were placed in the surface of Murashige & Skoog medium. (a) control, (b) T_1: 2-nonanone (abrupt system) and (c) T_2: 2-nonanone + Lanolin (controlled system). Error bars indicate error standard of mean (N=3). The letters indicate differences statistically significant according to ANOVA (LSD test, $p < 0.05$) (*ns= no significant difference).

6.3.3. Description of *L. sativa* leaf. Confocal laser microscopy was performed to evaluate anatomy of tissue and cell at leaf level to generate visual image of *L. sativa* exposed to 2-nonanone. The Fig.4a-f shows the leaf cell morphology indicating through qualitative aspect that cell wall shape (green color) were unchanged after applied treatments, while chloroplasts (red color) were observed with a sharper form when seedlings were exposed to 2-nonanone. Moreover, the number of open-stomata (surface: 420 x 298 µm) was increased when 2-nonanone was incorporated into lanolin matrix (Fig. 4g), observing a 275% of increase respect to control at 1.3 versus 5.0, respectively.

Furthermore, the length of stomata aperture tends to increase after the application of 2-nonanone with lanolin, but no statistically significant differences were found (Fig. 4h).

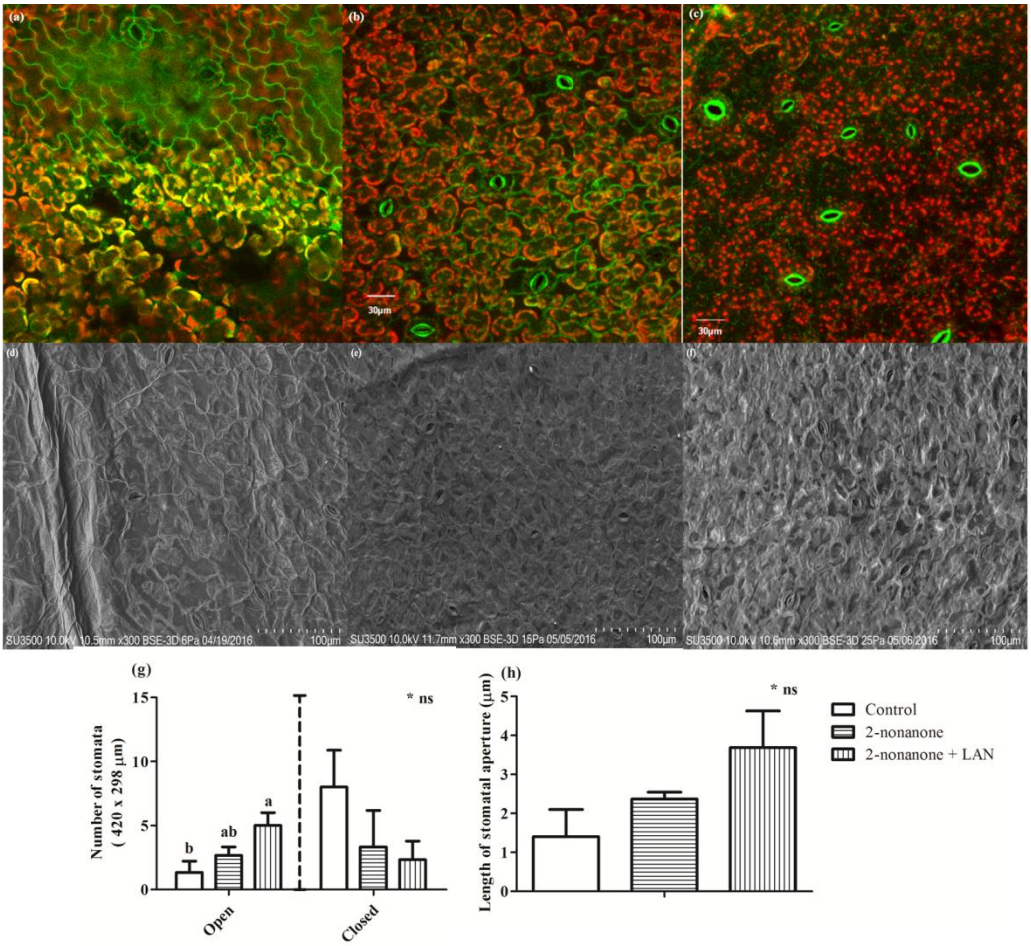


Figure 4. Representative photographs of leaf surface of *L. sativa* seedlings exposed to 2-nonanone (50 ppm) during 10 days. The *L. sativa* seedlings were placed in the surface of Murashige & Skoog medium: (a,d) control and exposed to T_1: 2-nonanone (abrupt system) (b,e) and T_2: 2-nonanone + Lanolin (controlled system) (c,f). Density of stomata (Number of stomata on surface of 420 x 298 µm) and length of stomatal aperture were evaluated. Error bars indicate error standard of mean (N=3). The letters indicate differences statistically significant according to ANOVA (LSD test, $p < 0.05$) (*ns= no significant difference).

6.3.4. Elemental microanalysis of root and leaf of *L. sativa* seedlings. The elemental distribution of elements was monitored through scanning electron microscopy coupled to X-ray elemental microanalysis. The three analyzed zones for mapping elements indicated that there is no alteration in the elements distribution on seedlings surface after volatile exposition. The Fig. 5 shows the description of elemental analyses carried out on the surface of *L. sativa* seedlings. The roots exposed to 2-nonanone was composed by carbon (48.6 - 59.8 %) and oxygen (36.7 - 43.7 %), principally. In minor percentage were found nitrogen, phosphorus and potassium (< 5%), independently of the treatment applied. The shoot composition was carbon (48.7 - 51.9%), oxygen (28%), nitrogen (8.4 - 9.7%), phosphorus (1.4 – 1.7 %), potassium (6.0 – 7.1 %). In minor percentage were found sulfur (0.6 - 0.9%) and chlorine (1.0 – 1.3 %). There were not significant differences among the treatments.

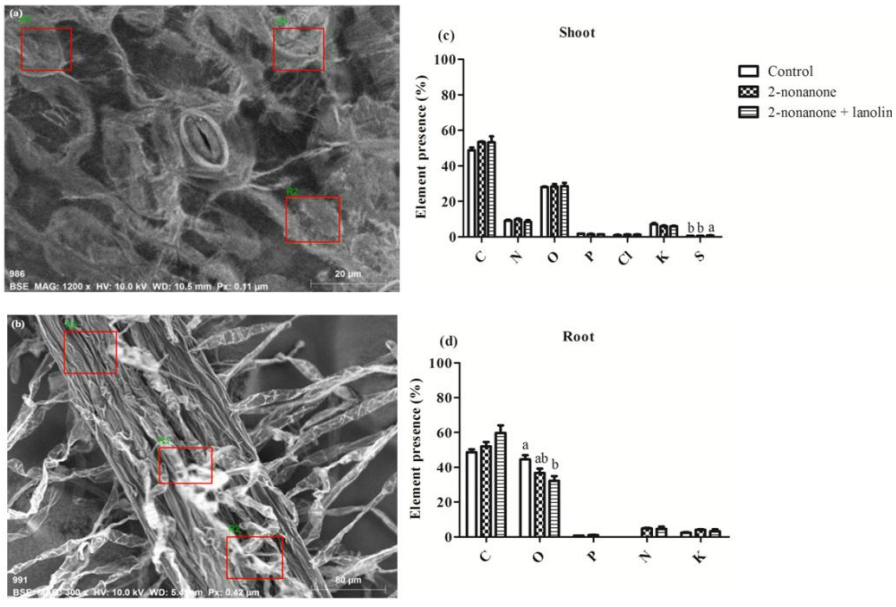


Figure 5. Percentage of elements on surface of *Lactuca sativa* seedlings at root and shoot level after 10 days of exposition to 2-nonanone (50 ppm). (a-b) Red squares are representative zones of leaf and root analyzed by Scanning electron microscopy coupled to X-ray elemental microanalysis. Percentages of elements presents on (c) leaf and (d) root after seedlings exposed to two delivery volatile systems: T_1: 2-nonanone (abrupt) and T_2: 2-nonanone + Lanolin (controlled). Mean ± standard deviation (N=3). (*ns= no significant difference).

6.4 DISCUSSION

VOCs are carbon-containing compounds with physico-chemical properties that facilitate their volatile emission making them ideal molecules to interact with "target organism" (Audrain et al., 2015). VOCs can diffuse through water, air and soil, so they play an ecological role at above and below- ground contributing in multitrophic interactions involving plants health (Bailly and Weisskopff, 2012; Bitas et al., 2013; Kanchiswamy et al., 2015). In the lasts years, VOCs have been intensively studied due to participate in physiological processes such as (1) plant tissue damaged by predators, (2) plant defense response, and (3) tolerance mediation of abiotic stress (T°, drought, salinity and light intensity) (Dudareva et al., 2013; Dong et al., 2016). However, in the last decade VOCs released by bacterial species have shown a great ability as a signal molecule that induce plant growth through changes at physiological level without physical contact, emerging as new sustainable tool for inducing plant growth (Kai et al., 2016). One of the first research (Ryu et al., 2003) reported that 2,3-butanediol elicited growth of surface leaf area of *A. thaliana*. Furthermore, 3-hydroxy-2-butanone and 2-pentylfuran induced the increase of fresh weight in tobacco and *A. thaliana*, respectively (Zou et al., 2010; Ann et al., 2013). In addition, dimethylhexadecylamine elicited the increase of fresh weight, stem length and root density on *M. sativa* and β-caryophyllene induced the increase of fresh weight, root length, shoot length and root density on *L. sativa* seedlings (Minerdi et al., 2011; Velázquez-Becerra et al., 2011).

Actions mechanisms associated with physiological effects related to growth induction have been reported in specific plant-bacteria interaction. Specifically, VOCs emitted by *B. subtilis* GB03 induced changes in genes belong to iron acquisition in *A. thaliana*,

stimulating chlorophyll content. Furthermore, *B. subtilis* strain JS produced changes in profile of gene expression on tobacco seedlings involving the following processes: metabolic processes, response to stimulus, cellular processes, reproduction, biological regulation, among others (Zhang et al., 2009; Kim et al., 2015). Nevertheless, the novel mechanisms that allow understand the role of specific VOCs on plants must be studied in different plant species. In this study we used 2-nonanone as bioactive VOC to prospect physiological effects on *L. sativa*, which have been scarcely studied so far.

The results demonstrated that 2-nonanone elicited the growth at root and shoot level on *L. sativa* seedlings independently of lanolin presence. However, lateral root length was increased under controlled delivery of 2-nonanone, suggesting that the release system of bioactive compound can be a critical factor in the radical development of *L. sativa*. In order to prospect external morphology of root, the scanning electron microscopy was used to reveal details of surface tissue surface to understand the possible physiological effect (Nylese et al., 2015). The root system plays an important role in plant support and acquisition of water and minerals (Casimiro et al.., 2003; Datta et al., 2011; Salazar-Henao et al., 2016). The results indicated that 2-nonanone elicited the increase of length and density of root hair on *L. sativa* seedlings, suggesting their relevant role to increase radical absorption zone and tolerance to both abiotic and biotic stress, providing the best conditions to environmental adaptation and exploratory capacity. Respect to physiological aspect, 2-nonanone can be involved in pathways associated with lateral root and root hair development, which is produced by hormones regulation and nutrients availability (Osmont et al., 2007; Hodge et al., 2009; Ruiz Herrera et al., 2015). It should be mentioned that epidermis is the tissue type that acts as barrier to the external environment with the

following functions: (1) mechanical protection against external agents, (2) limit transpiration and avoid excessive loss of water, (3) gas exchange regulation and (4) stimuli perception (Javelle et al., 2011). The results showed that 2-nonanone did not change both the morphology and size of root epidermal cells.

The leaf is the organ related with photosynthesis process, where the plant captures the light energy to produce vital chemical reactions. Microscopy analyses both scanning electron and confocal provided relevant evidence about external morphology with high resolution and magnification to visualize the surface structure (Pathan et al., 2008). Stomata are a small specialized cell localized on leaf surface formed by two specialized epidermal guard cells. The biological importance of stomata is the regulation of both gases flow and transpiration water loss (Lawson, 2009; Kollist et al., 2014). Our results indicated that *L. sativa* seedlings exposed to T_1 (2-nonanone + lanolin) increased the number of open stomata with a tendency to increase length of stomata aperture, suggesting that the controlled delivery of 2-nonanone can play an essential role to elicit the stomata aperture and gas exchange or could be the way how the volatile is incorporated by the plan and subsequently transported to the radical zone.

Additionally, the chlorophyll fluorescence was used to visualize chloroplast state (Iwai et al., 2015). Chloroplast is an organelle characterized by having chlorophyll-protein complexes associated with photosynthesis process; the data indicated that its morphology was not altered under 2-nonanone exposition. In addition, this technique allows the acquisition of cell wall imagen, providing evidence indicating that this organ was not altered after exposure to volatile. It should be mentioned that cell wall is a structure

characterized by providing rigidity, support and protection to the plant cell, allowing the passage of substances without controlled system (Gilbert, 2010).

To complement the information given by microscopic analysis about the surface morphology, an scanning electron microscopy coupled to X-ray elemental microanalysis was employed to perform a visual microcaracterization of intensity and distribution of elements present in *L. sativa* seedlings. The results indicated that 2-nonanone exposition does not change the composition of carbon, oxygen and macro elements in the surface of root and shoot, independently of delivery system of 2-nonanone (abrupt or controlled). The percentage of carbon and oxygen were found in the same quantity in all treatments respect to control at leaf and root level, these are characterized by being provided by water and assimilated biochemically through carboxylation and oxide-reduction reactions. In addition, NPK are found in same proportion in all treatments respect to control at both levels, indicating that 2-nonanone did not modify normal physiological processes associated with structural assimilation of nutrients. It noteworthy that nitrogen is a constituent of aminoacids, protein, nucleic acids and co-enzymes, while phosphorus and potassium are relevant nutrients that play an important role in energy storage and as enzyme cofactor respectively. It is noted that the presence of Cl in low concentration on leaf probably due to the proximity to the stomate, which use this element to regulate its aperture (Taiz and Zeiger, 2006).

In summary, the results presented above indicated that the application of 2-nonanone (abrupt delivery) had the ability to induce the growth on *L. sativa* seedlings by increasing the absorption surface of nutrients through the induction of length and root hair density without producing a radical morphological changes in cells. Furthermore, the mixture of 2-

nonanone with lanolin (controlled delivery) induced stomatal aperture, suggesting that 2-nonanone is perceived by this structure, in concordance with the model described by Widhalm et al. (2015). Furthermore, Matsui (2016) reported that VOCs from the atmosphere enter to intra cellular space (mesophyll) through stomata, indicating that VOCs are partitioned between gas and liquid phase inside the cell, depending on the physicochemical characteristics of the compound involved and metabolized in the cytosol. Nevertheless, studies about cellular processes and perception of volatiles should be performed to understand as a volatile compound is captured by the plant trigging a physiological action to induce growth. To our knowledge, this is the first study that provides evidence about the significant physiological effects of 2-nonanone on *L. sativa* seedlings exposed during 10 days through visual tools. Based on the above described, our findings show that VOCs can be an important tool to prospect new alternatives friendly to the environment for decreasing the application of chemical products.

.

CHAPTER VII:

General discussion

GENERAL DISCUSSION

VOCs are secondary metabolites considered by-products derived from primary metabolism so they are not essential to vital functioning, but they are documented as relevant chemical signals that allow communication involving intra and inter- organisms at both short and long distance acting as semio- or info-chemicals (Kanchiswamy et al., 2015ab). They are released by diverse bacterial species found in the soil ecosystem (Bitas et al., 2013; Kanchiswamy et al., 2015ab). The ability of bacterial VOCs to activate signaling network that contribute to induce growth through indirect (i.e antifungal, antimicrobial, antinematode and insecticidal) and direct (i.e hormone balance and nutrient availability) pathways have been studied in the last years (Zhang et al., 2007; Davis et al., 2013; Kim et al., 2013; Elkahoui et al., 2015; Xu et al., 2015). Approximately, 100 VOCs have been identified from bacterial species through gas chromatography coupled to mass spectrometry. However, the elucidation of chemical structures that induce plant growth by direct pathways is limited and few bioactive compounds have been documented to date (Kai et al., 2016). Under this context, the focus of the present thesis was to identify bacterial VOCs and subsequently expose *L. sativa* seedlings to them for evaluating growth inducing activity with the objective of performing the first and novel prospection for applying bacterial VOCs as new horticultural strategy for decreasing chemical product applications.

The *L. sativa* was proposed as model vegetable to test bacterial volatiles because they have an easy management, uniform germination, sensibility under environmental conditions and fast growth so it is widely used to test pure and mixtures of compounds (Bagur-González et al., 2010; Charles et al., 2011). In Chile, it constitutes the second most

cultivated vegetable, covering 6,272 ha (Eguillor-Recabarren and Acuña-Reyes, 2016). Specifically, *L. sativa* var. capitata (Green lettuce cv Reina de mayo asepo) was used in this study for greenhouse production and comprises the most traditional Chilean cultivar. Therefore, *L. sativa* seedlings were used as a "target" vegetable to elucidate novel VOCs for inducing growth and design a friendly alternative tool. The study was carried out in four stages: (1) prospection of soil bacterial species with growth inducing activity, (2) optimization of culture conditions for bacterial growing to release bioactive VOCs, (3) determination of VOCs involved to elicit growth and (4) elucidation of possible action mechanisms of bioactive VOC to induce *L. sativa* growth.

Thus, a soil bacteria scanning was performed through "Voges Proskauer test", which was used as quick tool for selecting soil bacterial species producing 3-hydroxy-2-butanone (acetoin), which is a compound widely reported as direct growth inducer and to trigger resistance against abiotic stress (Rudrappa et al., 2010; Ann et al., 2013). The implementation of this tool allowed select 10 soil bacterial species belonging to positive and negative Gram species such as *Bacillus*, *Staphylococcus* and *Serratia* were used in the first instance. Then, bacteria strains were subject for performing bioassays in bi-compartment Petri dishes to evaluate the capacity of bacterial VOCs for eliciting *L. sativa* growth. This experimental design allows only aerial contact (headspace) due to the presence of central partition, where two seedlings are placed in one of the compartments and a bacterial strain is inoculated in the second compartment (Bailly and Weisskopf, 2012; Kanchiswamy et al., 2015ab). It is worth mentioning that bacterial strains were placed in three culture media: 1) Murashige & Skoog, 2) nutrient and 3) Methyl Red & Voges Proskauer, because they have been widely used in previous reports as medium for bacterial

growth (Blom et al., 2011; Asari et al., 2016; Kai et al., 2016). Furthermore, the seedlings were exposed to bacterial VOCs for 7 and 10 days because there are evidences showing that the induction effects can be detected after this exposure period according to several reports (Zhang et al., 2007; Gutiérrez-Luna et al., 2010; Zou et al., 2010; minerdi et al., 2011).

The results indicated that *Bacillus* species have a higher ability to elicit growth at root and shoot level by VOCs emission after 7 and 10 days in the most of the tested culture media than Gram negative species as *Pseudomonas*, *Serratia* and *Staphylococcus*. The inducer growth effects attributed to VOCs released from *Bacillus* species have been documented in several reports, which have shown significant effects to induce the root development, including primary root length, lateral root number and lateral root length according to Gutiérrez-Luna et al. (2010). Furthermore, relevant results have been found at foliar level in parameters such as leaf surface area, true leaf and chlorophyll content (Ryu et al., 2003; Banchio et al., 2009; Zhang et al., 2009; Meldau et al., 2013). However, the differential effects within the same *Bacillus* species can be contributed to the divergence of volatile profiles, which is an intrinsic property derived from metabolic activity carried out differently for each strain belonging to the same bacterial species (Farag et al., 2006). Gram negative species, such as *Pseudomonas* species, have been scarcely reported to release VOCs with growth inducer activity. Santoro et al. (2011) showed that *P. fluorecens* elicited the increase of weight on *Menta Piperita*, and recently Park et al. (2015) reported that *P. fluorecens* SS101 induced the increase of weight on tobacco seedlings. Furthermore, there is a limited knowledge about *Serratia* and *Staphylococcus*, which only have been reported as growth inducers by Kai and Piechulla (2009) and Vespermann et al. (2007), respectively.

However, other Gram species negative have been reported with growth inducer activity as *B. ambifaria*, which have elicited the increase of lateral root number and shoot biomass on *A. thaliana* according to Groenhagen et al. (2013). Other work developed by Bailly et al. (2014), showed that *E. coli* emitted VOCs that induced growth on biomass and secondary roots in *A. thaliana*, whereas Bhattacharyya et al. (2015) indicated that *P. vulgaris* elicited the increase on fresh weight, root length, shoot length and number of lateral root in the same plant species mentioned above.

The results of this thesis about the effect of bacterial VOCs on *L. sativa* seedlings provide the evidence to propose them as growth inducers. Therefore, it was necessary to optimize the conditions for bacterial grown to evaluate the best conditions for obtaining the best activity of the bacterial volatiles. Different culture medium and both inoculums concentration and quantity were evaluated on the selected bacterium, *Bacillus* sp. BCT9, due to its great ability to induce *L. sativa* growth, compared with other evaluated bacterial strains. The results indicated that BCT9 grown in all culture media had the ability to induce growth on *L. sativa* seedlings, where a strong modulation effect was found in response to inoculum concentration applied, $5{,}0x10^{7}$ and $6{,}0x10^{7}$ CFU mL^{-1} elicited root growth and 2,7 and 2,0 $x10^{8}$ CFU/mL^{-1} elicited shoot growth, showing that bacterial concentration plays an important role for modulating growth, constituting a relevant parameter compared with culture medium. In contrast, the number of lateral roots and dry weight were increased independently from the used culture medium and concentration. However, differential effects on growth promotion were found when comparing all culture media, which may be produced by their composition and the capacity of BCT9 to multiply and grow cellularly, in agreeing with the report by Blom et al. (2011) and Asari et al. (2016) in others bacterial-

seedlings interactions. Methyl Red Voges Proskauer is composed by D-(+)-glucose, peptone and buffer phosphate whereas nutrient medium contained beef extract and peptone. Besides, Murashige & Skoog is a mineral medium with saccharose as carbon source (Bailly and Weisskopf, 2012). The data showed that BCT9 grows faster in Methyl Red Voges Proskauer in relation to Murashige & Skoog, but inverse relations in the cellular size were obtained. These results suggested that a quick growth of BCT9 and composition of culture medium may be more relevant compared with cell size to release a determinate concentration of bioactive volatiles for eliciting growth, evidencing that the induction of metabolism pathways (culture medium) is primordial in relation to surface (cell size) emission of VOCs. These results suggest that diferential complexity of culture media can influence strongly in the volatile emission due the metabolism energy of a bacterial species to hydrolyze complex polymers, so a direct availability of nutrients (bioavailability) allows that strains can have a favourable metabolism to release volatiles. Therefore, the direct source of carbon constitutes by glucose and aminoacids derived from peptone in conjunction with buffer phosphate, probably allows that BCT9 strain have a higher ability to release bioactive volatiles, compared with the others mediums tested as nutrient and Murashige & Skoog. Furthermore, inoculum quantity has been proposed as relevant parameter by Blom et al. (2011). Our results indicated that the increase of root length was elicited predominantly after 60 µl of applied inoculum when BCT9 was grown in Methyl Red & Voges Proskauer and Murashige & Skoog media whereas a number of lateral roots, dry weight and shoot length were increased after exposition to VOCs released by 15 and 30 µl of inoculums in all culture media. Agreeing with the reported by Blom et al. (2011), who showed that *B. pyrrocinia* Bcc171 elicited the increase of dry weight on *A. thaliana* when it was cultivated in Luria Bertani and Methyl Red Voges Proskauer media, achieving the best

yield when 10 µL of inoculums were applied. In addition, Velázquez-Becerra et al. (2011) reported a dose-dependence response of *M. sativa* exposed to VOCs released by *A. agilis* UMCV2, reaching the best increase growth after 50 µL of inoculum grown in nutrient medium. Recently, Asari et al. (2016) showed that VOCs emitted by *Bacillus* species induced a significant increase on dry weight of *A. thaliana* with doses of *B. amyloliquefaciens* UCMB5113 from 20 to 100 µL inoculated in Murashige & Skoog. These results have provided the suitable evidence that low doses of bacterial strains are optimal to elicit increasing growth, independent from bacterial strain and plant target involved in the interaction, being a phenomenon widely found according to the studies carried out so far.

Fort the identification of VOCs, Methyl Red & Voges Proskauer medium was selected, because BCT9 emit VOCs with greater growth-inducing effect when grown in this culture media. The volatile collection was performed through solid phase microextraction (SPME) and identified by gas chromatograph coupled to a mass spectrometer (Korpi et al., 2009). The identified VOCs are produced from two biosynthetic pathways: a) pyruvate fermentation and b) fatty acid cycle. In the first instance, the bioactive products (3-hydroxy-2-butanone and 2,3-butanediol) are derived from the condensation of two molecules of pyruvate into acetolactate, then descarboxylated to form 3-hydroxy-2-butanone (acetoin) and later, this compound is reduced to 2,3-butanediol. In the second instance, methyl-ketones (2-nonanone, 2-undecanone, 2-tridecanone and 2-pentadecanone) is originated from acetyl-CoA, which is a substrate that reacts with malonate to form fatty acids (Ryu et al., 2003; Schulz and Dickschat, 2007; Korpi et al., 2009). The data indicated that the bioactive volatiles elicited growth on *L. sativa* seedlings in at least one growth parameter at doses of 50 and 0.05 ppm with a greater effect to elicit growth at root and

shoot level (3-hydroxy-2-butanone, 2,3-butanediol, 2-nonanone, 2-undecanone, 2-tridecanone and 2-pentadecanone) while the induction of lateral root length is a more limited parameter induced specifically by 2-nonanone at 50 ppm. It should be mentioned that two systems are tested to evaluate *L. sativa* growth in response to an abrupt and controlled delivery. Lanolin is a wax matrix used to minimize the initial burst of VOCs into bioassays for controlling their release (Kessler and Baldwin, 2001; Groenhagen et al., 2013). The results indicated that similar effects on growth induction are found in both delivery systems, suggesting that receptor of seedlings can trigger growth induction in the presence of the compound independent from the delivery system. Furthermore, the results are consistent with the reports by other authors in respect to VOCs application for inducing growth, such as 2-pentylfuran, indole, dimethyl disulfide and acetophenone, which have been applied in low concentrations (Zou et al., 2010; Meldau et al., 2013; Bhattacharyya et al., 2015). This result suggested that lower doses are more effective to elicit growth at radical and foliar level, independent from compound chemical nature (Zou et al.,2010; Blom et al., 2011; Meldau et al., 2013; Ann et al., 2013; Bhattacharyya et al., 2015). Surprisingly, the higher concentration of methyl-ketones modulated strongly the leaf development, where as the number of carbon increases, the foliar growth increases, which has not been previously reported, suggesting that the compound structure (carbon number) may have relevance to induce a specific effect.

The 2-nonanone was selected for prospecting the action mechanism for eliciting growth, taking into account the relevant evidence to elicit radical and foliar growth on *L. sativa*. The 2-nonanone represents an interesting compound to be studied considering its ability to induce lateral root length, which is a measurement parameter to evaluate growth

promoting activity because the rapid establishing of lateral root is advantageous for seedlings (Nibau et al., 2008). The first approximation was performed through modern visual techniques that allow analyze fresh samples without destroying or treating the sample previously (Pathan et al., 2008; Gilbert, 2010; Nylese et al., 2015).

The evaluation of cell morphology through representative photographs indicated that seedlings exposed only to 2-nonanone increased significantly the number and length of radical hairs while the mixture of 2-nonanone and lanolin induced the aperture of stomata, suggesting that at cellular level the delivery system is essential to elicit these effects, giving a first focus to a possible action mechanism and signal pathways involved. The results indicated that 2-nonanone elicited the increase on root hair development, which is a plant organ with the role to uptake water and nutrients from soil solution as well as give a tolerance to environmental stresses, so the compound can play an essential role to adaptation and exploratory capacity. On the other hand, stomata opening after exposition to controlled delivery system, suggest their possible influence on gas flow and water loss through transpiration, whereby they can modulate photosynthesis process, but more evidence is needed to confirm this. The VOC response agrees with the model reported by Widhalm et al. (2015), who documented the role of stomata for trafficking on vegetal cell. On the other hand, the chloroplast fluorescence indicated that its morphology is not altered after compound exposition. Thus, the photosynthetic apparatus is not structurally altered according to this first approach.

In summary, *L. sativa* represented a good model vegetable to evaluate bacterial VOCs because it allowed growth parameter evaluation on seedling exposure to VOCs emitted by soil bacteria under controlled conditions. Likewise, this model allowed tracking soil

bacterial species and optimized culture conditions for emitting VOCs to elicit growth. Subsequently, seedlings target allow determining the effects of bioactive compounds in growth modulation to elucidate triggered action mechanisms. Finally, the important results found in this thesis are summarized in the following proposed scheme.

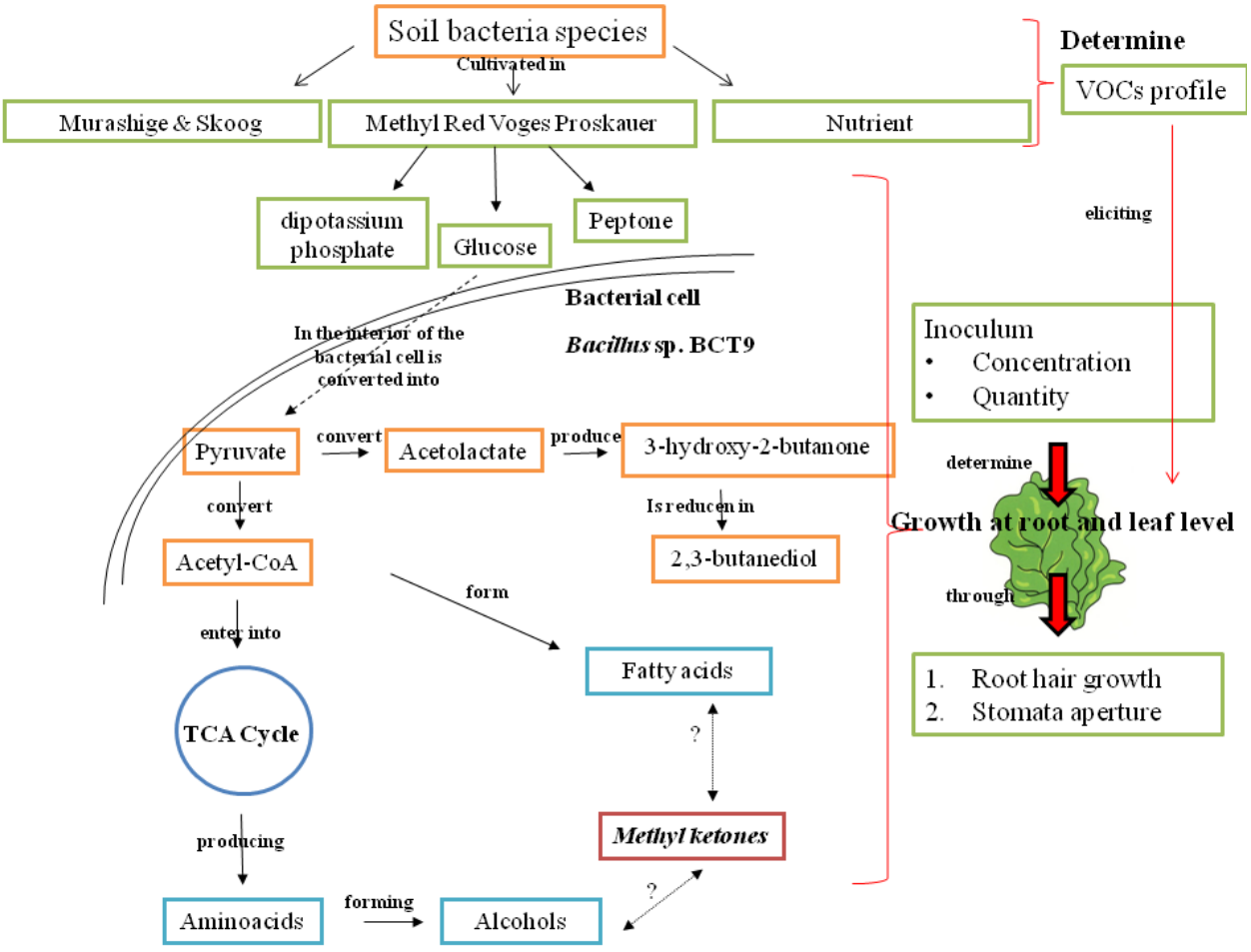


Figure 6.1. Thesis outline scheme about the study stages of the VOCs bioactive activity to elicit growth on *L. sativa* seedlings. Question marks indicate that methyl-ketones production not determined yet. Abbreviations: TCA= Tricarboxylic acid cycle, Acetyl-CoA= Acetyl-Coenzyme A, VOCs= Volatile Organic Compounds.

CONCLUDING REMARKS

The growth-inducing activity triggered by bacterial VOCs on *L. sativa* seedlings was showed under controlled conditions. In the first approach, diverse bacterial species belonging to *Bacillu*s, *Staphylococcus* and *Serratia* species isolated from soil were selected through the 3-hydroxy-2-butanone production, so they were employed to evaluate its VOCs emission for eliciting *L. sativa* growth. In the later approach, bacterial culture condition of selected strain (*Bacillus* sp. BCT9) was optimized to produce a volatile profile that led to better growth. In the third approach, the identification of bioactive VOCs was performed. Finally, the prospection of action mechanisms of 2-nonanone was carried out. Hence, the main conclusions from this thesis are:

- The first bioassay showed that strains belonging to *Bacillus* genus isolated from soil emitted volatiles with greater potential for inducing growth in *L. sativa* seedlings.
- Concentration and quantity of inoculums are critical parameters to modulate growth at root and shoot level in *L. sativa* seedlings.
- The 3-hydroxy-2-butanone, 2,3-butanediol and methyl-ketones modulated growth on *L. sativa* seedlings grown under controlled conditions.
- Finally, the experiment performed under laboratory conditions suggest that 2-nonanone induce the stomata aperture, indicating that this compound can diffuse into vegetal cell through this route. Likewise, the increase of root hair as response indicates the contribution of this compound to increase growth parameter due to eliciting the increase of nutrient and water uptake, contributing with the necessary elements to strengthen the seedling.
-

FUTURE DIRECTIONS

The results represent strong evidence that VOCs emitted by soil bacterial species can induce growth on vegetable species through the activation of physiological pathways. Likewise, the bioassays performed with pure compounds proved their biological activity for eliciting *L. sativa* growth. However, there is limited knowledge in the following aspects:

- The evaluation of VOCs on other vegetable species could be useful for their implementation as sustainable alternative to the environment for reducing the chemical products application because they induce growth on *L. sativa* at root and shoot level.

- The modulator activity found in response to bacterial VOCs suggests that physiological effects can be handled. Hence, to determine a specific VOCs profile can increase the efficiency to elicit growth of a determinate parameter (i.e shoot and root length).

- Cell mechanisms activated after VOCs exposition should be studied to determine receptor and secondary messengers involved in the perception of the chemical signal. This knowledge can be applied for developing a strategic and specific tool that allows efficient VOCs application (i.e root or leaf).

- Phytohormones pathways can be studied in seedlings exposed to VOCs. In this

sense, to analyze the modulation of target genes involved in phytohormone expression could be useful for finding activated action mechanisms, increasing the knowledge about application-field.

- The gene modulation related to metabolism, growth and cellular signals contributes to understand the target genes modulated by VOCs on vegetable species, contributing to give a strong explanation of triggered effects for eliciting growth.

REFERENCES

Angeles, G., Owens, S., Ewers, F., 2004. Fluorescence shell: A novel view of sclereid morphology with the confocal laser scanning microscope. Microsc. Res. Tech. 63, 282–288.

Ann, M., Cho, Y., Ryu, H., Kim, H., Park, K., 2013. Growth Promotion of Tobacco Plant by 3-hydroxy-2-Butanone from *Bacillus vallismortis* EXTN-1. Korean J Pestic Sci. 17, 388-393.

Asari, S., Matzén, S., Petersen, M., Bejai, S., Meijer, J., 2016. Multiple effects of *Bacillus amyloliquefaciens* volatile compounds: plant growth promotion and growth inhibition of phytopathogens. FEMS Microbiol. Ecol. 92, fiw070.

Audrain, B., Farag, M., Ryu, C., Ghigo, JM., 2015. Role of bacterial volatile compounds in bacterial biology. FEMS Microbiol. Rev. 39, 222-233.

Badri, DV., Weir, TL., Van der Lelie, D., Vivanco, JM., 2009. Rhizosphere chemical dialogues: plant–microbe interactions. Curr. Opin. Biotechnol. 20, 642–650.

Bagur-González, M., Estepa-Molina, C., Martín-Peinado, F., Morales-Ruano, S. 2010. Toxicity assessment using *Lactuca sativa* L. bioassays of the metal(loid)s As, Cu, Mn, Pb and Zn in soluble-in-water satured soil extracts from an abandoned mining site. J. Soils Sediments. 11, 281-289.

Bailly, A., Weisskopf, L., 2012. The modulating effect of bacterial volatiles on plant growth. Plant Signal Behav.7, 79-85.

Bailly, A., Groenhagen, U., Schulz, S., Geisler, M., Eberl, L., Weisskopf, L., 2014. The inter-kingdom volatile signal indole promotes root development by interfering with auxin signaling. Plant J. 80, 758-771.

Banchio, E., Xie, X., Zhang, H., Paré, P., 2009. Soil bacteria elevate essential oil accumulation and emissions in sweet basil. J. Agric. Food Chem. 57, 653-657.

Bennett, JW., Inamdar, AA., 2015. Are Some Fungal Volatile Organic Compounds (VOCs) Mycotoxins?. Toxins (Basel). 7, 3785-3804.

Bhattacharyya, P., Jha, D., 2012. Plant Growth- Promoting Rhizobacteria (PGPR): Emergence in agriculture. World J. Microbiol. Biotechnol. 28, 1327- 1350.

Bhattacharyya, D., Garladinne, M., Lee, Y., 2015. Volatile indole produced by rhizobacterium *Proteus vulgaris* JBLS202 stimulates growth of *Arabidopsis thaliana* through auxin, cytokinin, and brassinosteroid pathways. J. Plant Growth Regul. 34, 158–168.

Bitas, V., Kim, H., Bennett, J., Kang, S., 2013. Sniffing on microbes: Diverse roles of microbial volatile organic compounds in plant health. Mol. Plant Microbe Interact. 26, 835- 843.

Bitas, V., McCartney, N., Li, N., Demers, J., Kim, JE., Kim, HS., et al., 2015. *Fusarium Oxysporum* Volatiles Enhance Plant Growth Via Affecting Auxin Transport and Signaling. Front Microbiol. 6, 1248.

Blom, D., Fabbri, C., Connor, E., Schiestl, F., Klauser, D., Boller, T., et al., 2011. Production of plant growth modulating volatiles is widespread among rhizosphere

bacteria and strongly depends on culture conditions. Environ. Microbiol. 13, 3047-3058.

Bond, J., Donaldson, L., Hill, S., Hitchcock, K., 2008. Safranine fluorescent staining of wood cell walls. Biotech Histochem. 83, 161-171.

Bulgarelli, D., Schlaeppi, K., Spaepen, S., Ver Loren van Themat, E., Schulze-Lefert, P.,2013. Structure and functions of the bacterial microbiota of plants. Annu Rev Plant Biol. 64, 807-838.

Campos, D., Acevedo, F., Morales, E., Aravena, J., Amiard, V., Jorquera, M., et al ., 2014. Microencapsulation by spray drying of nitrogen-fixing bacteria associated with lupin nodules. World J. Microbiol. Biotechnol. 30, 2371- 2378.

Casimiro, I., Beeckman, T., Graham, N., Bhalerao, R., Zhang, H., Casero, P., Sandberg, G. and Bennett, M. 2003. Dissecting *Arabidopsis* lateral root development. Trends. Plant. Sci. 8, 165-171.

Castulo-Rubio, DY., Alejandre-Ramírez, NA., Orozco-Mosqueda, MC., Santoyo, G., Macías-Rodríguez, L., Valencia-Cantero, E., 2015. Volatile Organic Compounds Produced by the Rhizobacterium *Arthrobacter agilis* UMCV2 Modulate *Sorghum bicolor* (Strategy II Plant) Morphogenesis and *SbFRO1* Transcription In Vitro. J. Plant Growth Regul. 34, 611-623.

Charles, J., Sancey, B., Morin-Crini, N., Badot, P., Degiorgi, F., Trunfio, G., et al ., 2011. Evaluation of the phytotoxicity of polycontaminated industrial effluents using the

lettuce plant (*Lactuca sativa*) as a bioindicator. Ecotoxicol. Environ. Saf. 74, 2057–2064.

Chen, Y., Gozzi, K., Chai, Y., 2015. A bacterial volatile signal for biofilm formation. mBio. 6(3): e00392-15.

Chung, J,, Song, G., Ryu, C., 2015. Sweet scents from good bacteria: Case studies on bacterial volatile compounds for plant growth and immunity. Plant Mol. Biol. 90, 677-687.

Datta, S., Kim, C., Pernas, M., Pires, N., Proust, H., Tam, T., et al., 2011. Root hairs: development, growth and evolution at the plant-soil interface. Plant Soil. 346, 1–14.

Davis, TS., Crippen, TL., Hofstetter, RW., Tomberlin, JK. 2013. Microbial volatile emissions as insect semiochemicals. J. Chem. Ecol. 39, 840-859.

Dessaux, Y., Grandclément, C., Faure, D., 2016. Engineering the Rhizosphere. Trends Plant Sci. 21, 266-278.

Díaz de Villegas, M., Delgado, G., Rivas, M., Torres, E., Saura, M., 2011. Implementation of an *in vitro* bioassays as an indicator of the bionutrient FitoMas E. Cien. Inv. Agr. 38, 205- 210.

Ditengou, FA., Muller, A., Rosenkranz, M., Felten, J., Lasok, H., Van Doorn, MM., et al., 2015. Volatile signalling by sesquiterpenes from ectomycorrhizal fungi reprogrammes root architecture. Nat Commun. 6, 6279.

Dong, F., Fu, X., Watanabe, N., Su, X., Yang, Z., 2016. Recent advances in the emission and functions of plant vegetative volatiles. Molecules. 21, 124.

Dotaniya, ML., Meena, VD., 2015. Rhizosphere Effect on nutrient availability in soil and its uptake by plants: A review. Proc Natl Acad Sci, India, Sect B Biol. Sci. 85, 1-12.

Dudareva, N., Klempien, A., Muhlemann, J., Kaplan, I., 2013. Biosynthesis, function and metabolic engineering of plant volatile organic compounds. New Phytol. 198, 16–32.

Eguillor-Recabarren, P., Acuña-Reyes, D. Boletín de Hortalizas frescas: Julio 2016. ODEPA, Ministerio de Agricultura, Gobierno de Chile. 24p.

Elkahoui, S., Diébali, N., Yaich, N., Azaiez, S., Hammami, M., Essid, R., Limam, F. 2015. Antifungal activity of volatile compounds-producing *Pseudomonas* P2 strain against *Rhizoctonia solani*. World J. Microbiol. Biotechnol. 31, 175-185.

Ezquer, I., Li, J., Ovecka, M., Baroja-Fernández, E., Muñoz, FJ., Montero, M., et al., 2010. Microbial volatile emissions promote accumulation of exceptionally high levels of starch in leaves in mono- and dicotyledonous plants. Plant Cell Physiol. 51, 1674–1693.

Farag, M., Ryu, C., Sumner, L., Paré P., 2006. GC–MS SPME profiling of rhizobacterial volatiles reveals prospective inducers of growth promotion and induced systemic resistance in plants. Phytochemistry. 67, 2262–2268.

Fernández, V., Guzmán-Delgado, P., Graça, J., Santos, S., Gil, L., 2016. Cuticle structure in relation to chemical composition: Re-assessing the prevailing model. Front Plant Sci. 7: 427.

Filipiak, W., Sponring, A., Baur, M., Filipiak, A., Ager, C., Wiesenhofer, H., et al., 2012. Molecular analysis of volatile metabolites released specifically by *Staphylococcus aureus* and *Pseudomonas aeruginosa*. BMC Microbiol. 12, 113.

Fincheira, P., Venthur, H., Mutis, A., Parada, M., Quiroz, A., 2016. Growth promotion of *Lactuca sativa* in response to volatile organic compounds emitted from diverse bacterial species. Microbiol. Res.193, 39-47.

Fincheira, P., Parra, L., Mutis, A., Parada, M., Quiroz, A. 2017. Volatiles emitted by *Bacillus* sp. BCT9 act as growth modulating agents on *Lactuca sativa* seedlings. In preparation.

García-Casal, M. 2007. La alimentación del futuro: Nuevas tecnologías y su importancia en la nutrición de la población. An Venez Nutr. 20,108-114.

Gennari, P. 2015. FAO: Statistical Pocketbook World Food and Agriculture. Food and Agriculture Organization of the United Nations. 232p.

Gilbert, H., 2010. The Biochemistry and Structural Biology of Plant Cell Wall Deconstruction. Plant Physiol. 153, 444–455.

Goswami, D., Thakker, JN., Dhandhukia, PC., 2016. Portraying mechanics of plant growth promoting rhizobacteria (PGPR): A review. Cogent Food Agric. 2,1127500.

Groenhagen, U., Baumgartner, R., Bailly, A., Gardiner, A., Eberl, L., Schulz, S., et al., 2013. Production of bioactive volatiles by different *Burkholderia ambifaria* Strains. J. Chem. Ecol. 39, 892-906.

Gutiérrez-Luna, F., López, J., Altamirano, J., Valencia, E., Reyes de la Cruz, H., Macías, L., 2010. Plant growth- promoting rhizobacteria modulate root- system architecture in *Arabidopsis thaliana* through volatile organic compound emission. Symbiosis. 51, 75- 83.

Hao, HT., Zhao, X., Shang, QH., Wang, Y., Guo, ZH., Zhang, YB., et al., 2016. Comparative digital gene expression analysis of the Arabidopsis response to volatiles emitted by *Bacillus amyloliquefaciens*. PLoS ONE. 11, e0158621.

Hodge, A., Berta, G., Doussan, C., Merchan, F., Crespi, M., 2009. Plant root growth, architecture and function. Plant Soil. 321, 153–187.

Hung, R., Lee, S., Bennett, JW., 2013. *Arabidopsis thaliana* as a model system for testing the effect of *Trichoderma* volatile organic compounds. Fungal Ecol. 6,19-26.

Insam, H., Seewald, M., 2010.Volatile organic compounds (VOCs) in soils. Biol. Fertil. Soils. 46,199–213.

Iwai, M., Yokono, M., Nakano, A., 2015. Toward understanding the multiple spatiotemporal dynamics of chlorophyll fluorescence. Plant Signal Behav.10, e1022014.

Jacobsen, S., Sorensen, M., Pedersen, S., Weiner, J., 2013. Feeding the world: genetically modified crops versus agricultural biodiversity. Agron Sustain Dev. 33, 651-662.

Javelle, M., Vernoud, V., Rogowsky, P., Ingram, G., 2011. Epidermis: the formation and functions of a fundamental plant tissue. New Phytol. 189, 17–39.

Jones, D., Taylor, W., Thornton, J., 1992. The rapid generation of mutation data matrices from protein sequences. CABIOS. 8, 275-82.

Kai, M., Piechulla, B., 2009. Plant growth promotion due to rhizobacterial volatiles - An effect of CO_2?. FEBS Lett. 583, 3473-3477.

Kai, M., Effmert, U., Piechulla, B., 2016. Bacterial-Plant-Interactions: Approaches to unravel the biological function of bacterial volatiles in the rhizosphere. Front Microbiol. 7, 108.

Kanchiswamy, C., Malnoy, M., Maffei, M., 2015a. Bioprospecting bacterial and fungal volatiles for sustainable agriculture. Trends Plant Sci. 20, 206-211.

Kanchiswamy, CN., Malnoy, M., Maffei, M. 2015b. Chemical diversity of microbial volatiles and their potential for plant growth and productivity. Front Plant Sci. 6, 151.

Kessler, A., Baldwin, I. 2001. Defensive function of herbivore-induced plant volatile emissions in nature. Science. 291, 2141-2144.

Kim, SA., Guerinot, ML. 2007. Mining iron: iron uptake and transport in plants. FEBS Lett. 581, 2273-2280.

Kim, KS., Lee, S., Ryu, CM. 2013.Interspecific bacterial sensing through airborne signals modulates locomotion and drug resistance. Nat Commun. 4, 1809.

Kim, J., Lee, J., Seo, SG., Lee, C., Woo, S., Kim, S., 2015. Gene expression profile affected by volatiles of new plant growth promoting rhizobacteria, *Bacillus subtilis* strain JS, in tobacco. Genes Genom. 37,387-397.

Kodama, Y., 2016. Time gating of chloroplast autofluorescence allows clearer fluorescence imaging in planta. PLoS ONE. 11:e0152484.

Kollist, H., Nuhkat, M., Roelfsema, M., 2014. Closing gaps: linking elements that control stomatal movement. New Phytol. 203, 44–62.

Korpi, A., Järnberg, J., Pasanen, AL., 2009. Microbial volatile organic compounds. Crit. Rev. Toxicol. 39,139–193.

Kwon, YS., Ryu, C., Lee, S., Park, HB., Han, KS., Lee, JH., et al., 2010. Proteome analysis of Arabidopsis seedlings exposed to bacterial volatiles. Planta. 232, 1355-1370.

Lawson, T., 2009. Guard cell photosynthesis and stomatal function. New Phytol. 181, 13–34.

Lee, B., Farag, MA., Park, HB., Kloepper, JW., Lee, SH., Ryu, CM., 2012. Induced Resistance by a Long-Chain Bacterial Volatile: Elicitation of Plant Systemic Defense by a C13 Volatile Produced by *Paenibacillus polymyxa*. PLos ONE. 7, e48744.

Marquez-Villavicencio, M., Weber, B., Witherell, R., Willis, D., Charkowski, A., 2011. The 3-hydroxy-2-butanone pathway is required for *Pectobacterium carotovorum* pathogenesis. PLoS ONE. 6, e22974.

Matsui, K., 2016. A portion of plant airborne communication is endorsed by uptake and metabolism of volatile organic compounds. Curr. Opin. Plant Biol. 32, 24–30.

Meldau, D., Meldau, S., Hoang, L., Underberg, S., Wünsche, H., Baldwin, I., 2013. Dimethyl disulfide produced by the naturally associated bacterium *Bacillus* sp B55 promotes *Nicotiana attenuata* growth by enhancing sulfur nutrition. Plant Cell. 25, 2731-2747.

Mendes, R., Garbeva, P., Raaijmakers, JM., 2013. The rhizosphere microbiome: significance of plant beneficial, plant pathogenic, and human pathogenic microorganisms. FEMS Microbiol. Rev. 37, 634–663.

Minerdi, D., Bossi, S., Maffei, M., Gullino, M., Garibaldi, A., 2011. *Fusarium oxysporum* and its bacterial consortium promote lettuce growth and expansin A5 gene expression through microbial volatile organic compounds (MVOC) emission. FEMS Microbiol. Ecol. 76, 342 - 351.

Nellemann, C., MacDevette, M., Manders, T., Eickhout, B., Svihus, B., Prins, A. G., Kaltenborn, B. P. 2009.The environmental food crisis – The environment's role in averting future food crises. A UNEP rapid response assessment.Norway.104 p.

Newell-McGloughlin, M. 2008. Nutritionally improved agricultural crops. Plant Physiol. 147, 939-953.

Nibau, C., Gibbs, D. and Coates, C. 2008. Branching out in new directions: the control of root architecture by lateral root formation. New Phytol. 179, 595- 614.

Nichols, M., Hilmi, M. 2009. Growing vegetables for home and market. FAO Diversification Booklet. Food and Agriculture Organization of the United Nations. 91 p.

Nylese, T., Berry, A., Oscher, S., 2015. Elemental analysis of silicon in plant material with variable-pressure SEM. Micros Today. 23,26-31.

Orozco-Mosqueda, M., Velázquez-Becerra, C., Macías-Rodríguez, L., Santoyo, G., Flores-Cortez, I., Alfaro-Cuevas, R., et al ., 2013. *Arthrobacter agilis* UMCV2 induces iron acquisition in *Medicago truncatula* (strategy I plant) in vitro via dimethylhexadecylamine emission. Plant Soil. 362, 51–66.

Ortíz-Castro, R., Contreras, H., Macías, L., López, J., 2009. The role of microbial signals in plant growth and development. Plant Signal Behav. 4, 701-712.

Osmont, K., Sibout, R., Hardtke, C., 2007. Hidden branches: Developments in root system architecture. Annu Rev Plant Biol. 58, 93-113.

Paul, D., Park, KS., 2013. Identification of Volatiles Produced by *Cladosporium cladosporioides* CL-1, a Fungal Biocontrol Agent That Promotes Plant Growth. Sensors (Basel). 13, 13969–13977.

Park, Y., Dutta, S., Ann, M., Raaijmakers, J., Park, K., 2015. Promotion of plant growth by *Pseudomonas fluorescens* strain SS101 via novel volatile organic compounds. Biochem. Biophys. Res. Commun. 461, 361-365.

Parra, L., Mutis, A., Ceballos, R., Lizama, M., Pardo, F., Perich, F., et al., 2009. Volatiles released from *Vaccinium corymbosum* were attractive to *Aegorhinus superciliosus*

(Coleoptera: Curculionidae) in an olfactometric bioassay. Environ. Entomol. 38, 781- 789.

Parvathi, A., Krishna, K., Jose, J., Joseph, N., Nair, S., 2009. Biochemical and molecular characterization of *Bacillus pumilus* isolated from coastal environment in cochin, India. Braz. J. Microbiol. 40, 269-275.

Pathan, AK., Bond, J., Gaskin, RE., 2008. Sample preparation for scanning electron microscopy of plant surfaces-Horses for courses. Micron. 39, 1049-1061.

Peace, T., Brock, K., Stills, H., 1994. Comparative analysis of the16s rRNA gene sequence of the putative agent of proliferative ileitis of hamsters. Int. J. Syst. Bacteriol. 44, 832–835.

Pem, D., Jeewon, R. 2015. Fruit and Vegetable Intake: Benefits and Progress of Nutrition Education Interventions- Narrative Review Article. Iran. J. Public Health. 44, 1309-1321.

Philippot, L., Raaijmakers, JM., Lemanceau, P., Van der Putten, WH., 2013. Going back to the roots: the microbial ecology of the rhizosphere. Nat. Rev. Microbiol. 11, 789-799.

Piechulla, B., Degenhardt, J., 2014. The emerging importance of microbial volatile organic compounds. Plant Cell Environ. 37, 811-812.

Ramírez, K., Lauber, C., Fierer, N., 2009. Microbial consumption and production of volatile organic compounds at the soil-litter interface. Biogeochemistry. 99, 97-107.

Romick, T., Fleming, H., 1998. Acetoin production as an indicator of growth and metabolic inhibition of *Listeria monocytogenes*. J Appl Microbiol. 84, 18-24.

Rudrappa, T., Biedrzycki, ML., Kunjeti, S., Donofrio, N., Czymmek, K., Paré, P., et al., 2010. The rhizobacterial elicitor acetoin induces systemic resistance in *Arabidopsis thaliana*. Commun Integr Biol. 3, 130-138.

Ruiz-Herrera, LF., Shane, M., López-Bucio, J., 2015. Nutritional regulation of root development. Wiley Interdiscip Rev Dev Biol. 4, 431–443.

Ryu, C., Farag, M., Hu, C., Reddy, M., Wei, H., Paré, P., 2003. Bacterial volatiles promote growth in *Arabidopsis*. Proc. Natl. Acad. Sci. U.S.A. 100, 4927- 4932.

Salazar-Henao, J., Vélez- Bermúdez, I., Schmidt, W., 2016. The regulation and plasticity of root hair patterning and morphogenesis. Development. 143, 1848-1858.

Sánchez-López, AM., Baslam, M., De Diego, N., Muñoz, FJ., Bahaji, A., Almagro, G.,et al., 2016. Volatile compounds emitted by diverse phytopathogenic microorganisms promote plant growth and flowering through cytokinin action. Plant Cell Environ. pce.12759.

Santoro, M., Zygadlo, J., Giordano, W., Banchio, E., 2011. Volatile organic compounds from rhizobacteria increase biosynthesis of essential oils and growth parameters in peppermint (*Mentha piperita*). Plant Physiol. Biochem. 49, 1177-1182.

Savci, S., 2012. Investigation of effect of chemical fertilizers on environment. APCBEE Procedia. 1, 287-292.

Schulz, S., Dickschat, J., 2007. Bacterial volatiles: the smell of small organisms. Nat Prod Rep. 24, 814-842.

Silva Dias, J. 2012. Nutritional quality and health benefits of vegetables: A review. Food Nutr Sci. 3, 1354-1374.

Sun, J., Zhang, L., Rao, B., Shen, Y., Wei, D., 2012. Enhanced acetoin production by *Serratia marcescens* H32 with expression of a water-forming NADH oxidase. Bioresour. Technol. 119, 94–98.

Taiz, L., Zeiger, E., 2006. Plant Physiology. 4th ed. Universitat Jaume I.

Tajima, R., Kato, Y., 2013. A quick method to estimate root length in each diameter class using freeware ImageJ. Plant Prod. Sci. 16, 9-11.

Tampe, J., Parra, L., Huaiquil, K., Quiroz, A., 2016. Potential repellent activity of the essential oil of *Ruta chalepensis* (Linnaeus) from Chile against *Aegorhinus superciliosus* (Guérin) (Coleoptera: Curculionidae). J. Soil Sci. Plant Nutr. 16, 48-59.

Tamura, K., Stecher, G., Peterson, D., Filipski, A., Kumar, S., 2013. MEGA6: Molecular evolutionary genetics analysis version 6.0. Mol. Biol. Evol. 30: 2725-2729.

Torres, M., 2003. Empleo de los ensayos con plantas en el control de contaminantes tóxicos ambientales. Rev Cubana Hig Epidemiol. 41, 2-3.

Vaishnav, A., Kumari, S., Jain, S., Varma, A., Choudhary, DK., 2015. Putative bacterial volatile-mediated growth in soybean (*Glycine max* L. Merrill) and expression of induced proteins under salt stress. J Appl Microbiol. 119, 539-551.

Valerio, ME., García, J., Peinado, FM., 2007. Determination of phytotoxicity of soluble elements in soils, based on bioassay with lettuce (*Lactuca sativa* L.). Sci. Total Environ. 378, 63-66.

Van Loon, L., 2007. Plant responses to plant growth-promoting rhizobacteria. Eur. J. Plant Pathol. 119, 243–254.

Van Dam, NM., Bouwmeester, HJ., 2016. Metabolomics in the Rhizosphere: Tapping into Belowground Chemical Communication. Trends Plant Sci. 21, 256-265.

Vejan, P., Abdullah, R., Khadiran, T., Ismail, S., Nasrulhaq Boyce, A., 2016. Role of plant growth promoting rhizobacteria in agricultural sustainability-A Review. Molecules. 21, E573.

Velázquez-Becerra, C., Macías, L., Bucio, J., Altamirano, J., Flores, I., Valencia E., 2011. A volatile organic compound analysis from *Arthrobacter agilis* identifies dimethylhexadecylamine, an amino- containing lipid modulating bacterial growth and *Medicago sativa* morphogenesis in vitro. Plant Soil. 339, 329- 340.

Venturi, V., Keel, C., 2016. Signaling in the rhizosphere. Trends Plant Sci. 21, 187-198.

Vespermann, A., Kai, M., Piechulla, B., 2007. Rhizobacterial volatiles affect the growth of fungi and *Arabidopsis thaliana*. Appl. Environ. Microbiol. 73, 5639- 5641.

Waldvogel-Abramowski, S., Waeber, G., Gassner, C., Buser, A., Frey., BM., Favrat, B., et al., 2015. Physiology of iron metabolism. Transfus Med Hemother. 41, 213–221.

Ward, MH., 2009. Too much of a good thing? Nitrate from nitrogen fertilizers and cancer. Rev Environ Health. 24, 357-363.

Widhalm, J., Jaini, R., Morgan, J., Dudareva, N., 2015. Rethinking how volatiles are released from plant cells. Trends Plant Sci. 20, 545-550.

Xiao, Z., Xu, P., 2007. Acetoin metabolism in bacteria. Crit. Rev. Microbiol. 33, 127–140.

Xiao, Z., Wang, X., Huang, Y., Huo, F., Zhu, X., Xi, L., et al., 2012. Thermophilic fermentation of acetoin and 2,3-butanediol by a novel Geobacillus strain. Biotechnol Biofuels. 6, 88.

Xie, X., Zhang, H., Paré P., 2009. Sustained growth promotion in *Arabidopsis* with long-term exposure to the beneficial soil bacterium *Bacillus subtilis* (GB03). Plant Signal Behav. 4, 948-953.

Xu, YY., Lu, H., Wang, X., Zhang, KQ., Li-GH. 2015. Effect of volatile organic compounds from bacteria on nematodes. Chem. Biodivers. 12, 1415-1421.

Zahid, M., Kaleem Abbasi, M., Hameed, S., Rahim, N., 2015. Isolation and identification of indigenous plant growth promoting rhizobacteria from Himalayan region of Kashmir and their effect on improving growth and nutrient contents of maize (*Zea mays* L.). Front Microbiol. 17, 207.

Zaman, M., Kurepin, L., Catto, W., Pharis, R., 2015. Enhancing crop yield with the use of N-based fertilizers co-applied with plant hormones or growth regulators. J. Sci. Food Agric. 95, 1777-1785.

Zhang, H., Kim, M., Krishnamachari, V., Payton, P., Sun, Y., Grimson, M., et al., 2007. Rhizobacterial volatile emissions regulate auxin homeostasis and cell expansion in *Arabidopsis*. Planta. 226, 839- 851.

Zhang, H., Xie, X., Kim, M., Kornyeyev, D., Holaday, S., Paré, P., 2008. Soil bacteria augment Arabidopsis photosynthesis by decreasing glucose sensing and abscisic acid levels *in planta*. Plant J. 56, 264–273.

Zhang, H., Sun, Y., Xie, X., Kim, M., Dowd, S., Paré, P., 2009. A soil bacterium regulates plant acquisition of iron via deficiency-inducible mechanisms. Plant J. 58, 568-577.

Zou, C., Li, Z., Yu, D., 2010. *Bacillus megaterium* Strain XTBG34 promotes plant growth by producing 2-pentylfuran. J. Microbiol. 48, 460-466.

APPENDICES

Appendix 1.

Table 1. Carbohydrate fermentation of Bacillus isolates (non-selected for bioassays) using API 50CH strips after 48 h. (+ = positive reaction; - = negative reaction; +/- = medium reaction)

Substrate	***Bacillus* strains**				
	BCT74-2	**BCT21**	**BCT11**	**BCT74-1**	**BCT5**
Temoin	-	-	-	-	-
glycerol	+	+/-	+	+/-	+/-
erythritol	-	-	-	-	-
D-arabinose	-	+	-	-	-
L-arabinose	+	+	+	+	+/-
D-ribose	+	+	+	+	+/-
D-xylose	-	+/-	-	+/-	+/-
L-xylose	-	-	-	-	-
D-adonitol	-	-	-	-	-
methyl-βD-xylopyranoside	-	-	-	-	-
D-galactose	-	+/-	-	+/-	+/-
D-glucose	+	+	+	+	+
D-fructose	+	+	+	+	+
D-mannose	+	+	+	+	-
L-sorbose	-	-	-	-	-
L-rhamnose	-	-	-	-	-
Ducitol	-	-	-	-	-
inositol	+	-	+	-	+/-
D-mannitol	+	+/-	+	+	+/-
D-sorbitol	+	-	+	+	-
methyl-αD-mannopyranoside	-	-	-	+/-	-
methyl-αD-glucopyranoside	+	-	+	+/-	-
N-acetylglucosamine	-	-	-	+/-	+
Amygdaline	+/-	-	-	+/-	+/-
Arbutine	+/-	+	-	+	+/-
Esculine citrato de fer	+/-	+/-	+/-	+/-	+/-
Salcine	+	+	+	+	+
D-celiobiose	+	+/-	+	+	+/-
D-maltose	+	-	+	+/-	+
D-lactose (origine bovine)	+/-	-	-	-	-
D-melibiose	-	-	-	+/-	+
D-saccharose	+	+/-	+	+	+
D-trehalose	+	-	+	+	+

Inuline	-	-	-	-	-
D-melezitose	-	-	-	-	-
D-raffinose	-	-	-	-	+
Almidon	+	-	+	-	+
Glycogene	+	-	+	-	+
Xylitol	-	-	-	-	-
Gentiobiose	+/-	-	+/-	+/-	+/-
D-turanose	-	-	-	+/-	-
D-lyxose	-	-	-	-	-
D-tagatose	-	+/-	-	+	-
D-fucose	-	-	-	-	-
L-fucose	-	-	-	-	-
D-arabitol	-	-	-	-	-
L-arabitol	-	-	-	-	-
Potassium gluconate	-	-	-	-	-
Potassium 2-cetogluconate	-	-	-	-	-
Potassium 5-cetogluconate	-	-	-	-	-

Appendix 2.

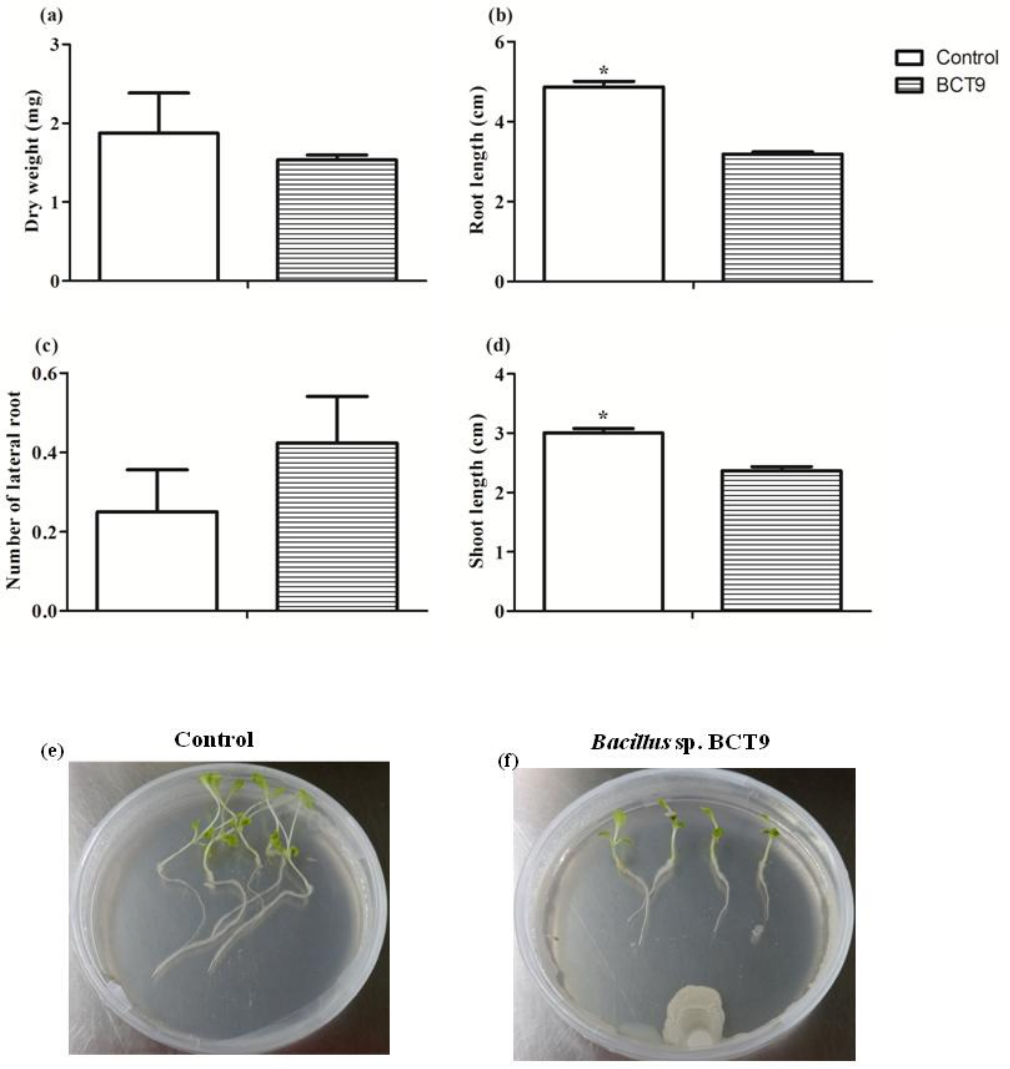


Figure 1. *L. sativa* growth in response to non-volatile metabolites (diffusion bioassays) emitted by *Bacillus* sp. BCT9 ($2.7x10^8$ CFU/mL) on day-10. *L. sativa* seedlings were placed in Petri dish with Murashige & Skoog medium. Plates non-inoculated were considered as control. The measurement parameters were (a) dry weight, (b) root length, (c) number of lateral root and (d) shoot length. Asterisks indicated significant differences as determined T-student test ($p < 0.05$). Bars represent standard error values (n=40)

Appendix 3.

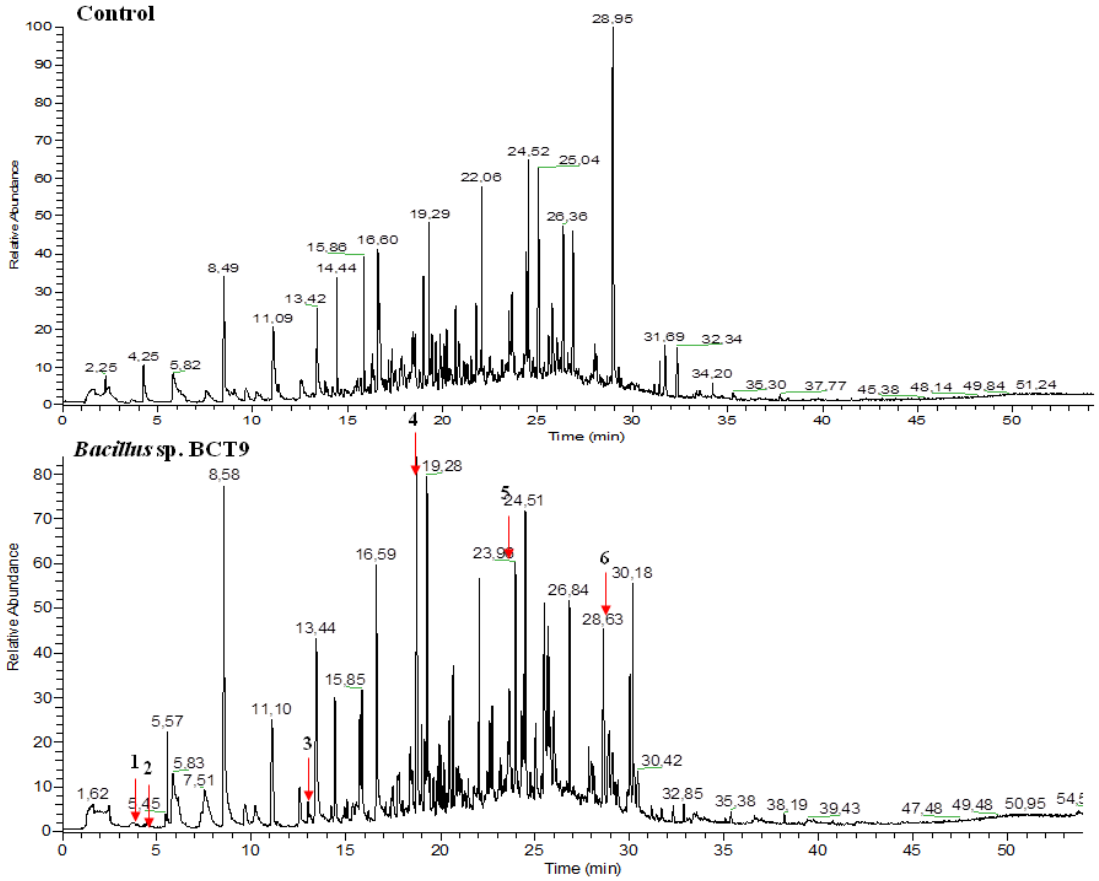


Figure 2. Chromatographic profiles of volatiles released by *Bacillus* sp. BCT9, compared with an uninoculated medium control (Methyl Red & Voges Proskauer). Identified compounds included 3-hydroxy-2-butanone [1], 2,3-butanediol [2], 2-nonanone[3], 2-undecanone [4], 2-tridecanone [5] and 2-pentadecanone (n=3).

Appendix 4.

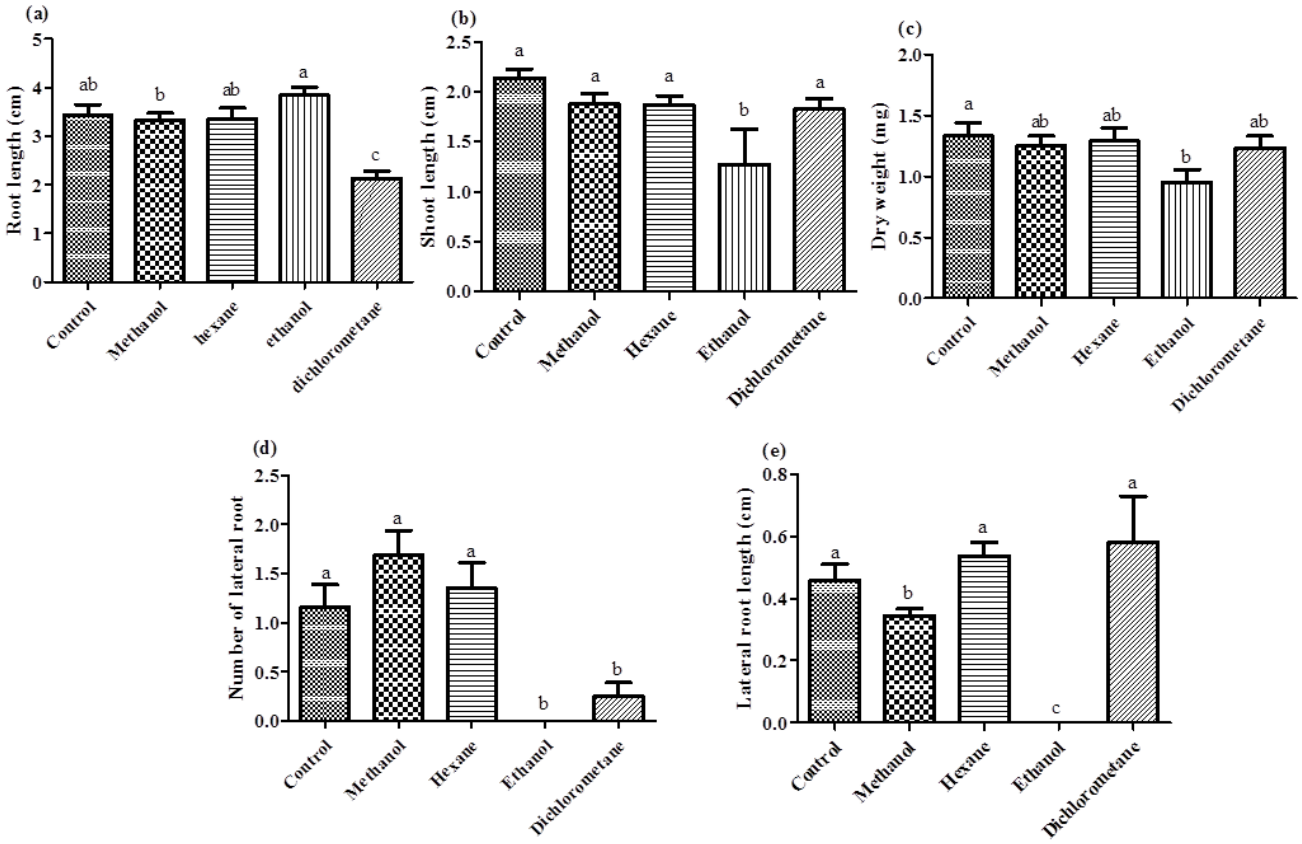


Figure 3. Evaluation of toxicity on *L. sativa* growth after expose to solvents on day-10. (a) root length, (b) Shoot length, (c) dry weight, (d) number of lateral root and (e) lateral root length. Letters indicate statistically significance differences according ANOVA test (LSD). Bars represent standard error. ns= no significant difference. ($p < 0.05$, n=18-20).

Appendix 5.

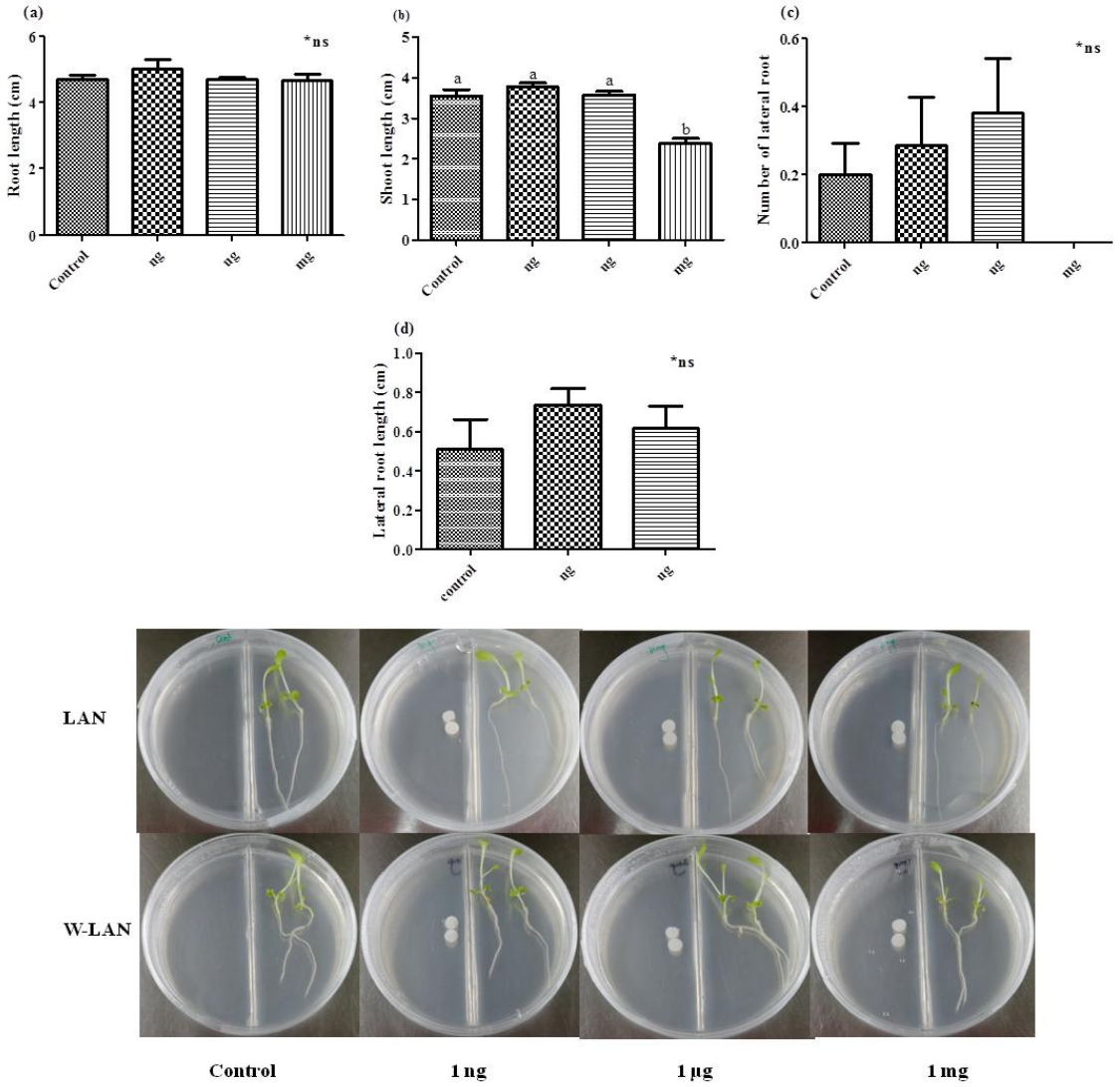


Figure 4. Growth induction on agronomical parameters of *L. sativa* seedlings after 10 days of applied mixtures of bioactive volatile compounds (3-hydroxy-2-butanone, 2,3-butanediol, 2-nonanone, 2-undecanone, 2-tridecanone and 2-pentadecanone) according to chromatogram proportion. Letters indicated significant differences according to LSD test ($P< 0.05$; n= 18-20). Bars indicated standard error. ns= no significant difference.

GENERAL PRODUCTIVITY

Conferences:

2016 Fincheira, P., Quiroz, A. Crecimiento de *L. sativa* modulado por compuestos orgánicos volátiles emitidos por diversas bacterias de suelo. III Taller Latinoamericano de PGPR y 2do workshop en Biotecnología y Medio Ambiente. Pucón-Chile.

2015 Fincheira, P., Mendez, L., Mutis, A., Parada, M and Quiroz, A. Volatile organic compounds emitted by *Bacillus subtilis* BCT9. V International Workshop "Advances in science and Technology of Bioresources. Pucón - Chile.

2014 Fincheira, P and Quiroz, A. Effects of volatile organic compounds emitted by soil bacteria on growth promotion of *Lactuca sativa* L. Congreso Latinoamericano de Química Ecológica. Bogota – Colombia.

2014 Fincheira, P., Heisse, M and Quiroz, A. Efecto de compuestos volátiles emitidos por bacterias de suelo en la promoción de crecimiento de *Lactuca sativa.* XX Congreso Latinoamericano de Microbiología. Cartagena de Indias- Colombia.

2013 Fincheira, P., Parra, L., Parada, M. and Quiroz, A. Effects of volatile organic compounds emitted by bacteria bct34 in *Arabidopsis thaliana* growth. IV International Workshop "Advances in science and Technology of Bioresources. Pucón - Chile.

2013 Jimenez, D., Cravero, M., Aedo, F., Muñoz, J., **Fincheira, P**., Quiroz, A. and Parra, L. Efecto promotor del crecimiento vegetal mediado por COVs emitidos por la bacteria bct90 aislada de lodos. Workshop Internacional y taller nacional "Valorizacion de Residuos Oportunidad para la innovación". Pucón- Chile.

2013 Fincheira, P., Parada, M. and Quiroz, A. Volatile Organic compounds emitted by soil microorganism with potential use in agriculture. II International symposium: Organic Matter Management & compost use in horticulture, III Workshop in Bioproducts for agriculture and III Iberoamerican Network for Biological Fertilizers for Agriculture Workshop. Santiago- Chile.

2013 Parada, M., Quiroz, A., Parra, L. and **Fincheira, P**. Efecto de volátiles emitidos por la bacteria bct34 en la promoción de crecimiento de *Arabidopsis thaliana*. II Conferencia Iberoamericana de Interacciones Beneficiosas Microorganismos- Planta-Ambiente. Sevilla-España.

Papers:

Fincheira, P., Venthur, H., Mutis, A., Parada, M., Quiroz, A. 2016. Growth promotion of *Lactuca sativa* in response to volatile organic compounds emitted from diverse bacterial species. Microbiological Research. 193, 39-47.

Fincheira, P., Parra, L., Mutis, A., Parada, M., Quiroz, A. 2017. Volatiles emitted by *Bacillus* sp. BCT9 act as growth modulating agents on *Lactuca sativa* seedlings. Microbiological Research. (envied).

Fincheira, P., Parada, M., Quiroz, A. 2017. Volatile organic compounds stimulate plant growing and seed germination of *Lactuca sativa*. Journal of Soil Science and Plant Nutrition. (envied).

Fincheira, P., Parada, M., Quiroz, A. 2017. Physiological response of *Lactuca sativa* exposed to 2-nonanone. Plant Physiology and Biochemistry. (envied).

Fincheira, P., Quiroz, A. 2017. Microbial volatiles as plant growth inducers. Microbiological Research. (envied).

Patent:

2017 Composición promotora de crecimiento de plántulas, un kit, un método de aplicación, uso de los compuestos orgánicos volátiles que comprenden la composición. Organización Internacional de la Organización Mundial de la Propiedad Intelectual. N° solicitud: PCT/IB2017/050793. Recepción: 13 de febrero 2017.

Adjudicated Project:

2016 Adjudicación de Proyecto VIU (Primera etapa). Encapsulado como inductor del crecimiento de lechuga (*Lactuca sativa* L) basado en compuestos orgánicos volátiles. Sexto concurso 2016 del programa de valorización de la investigación en la universidad del fondo de fomento al desarrollo científico y tecnológico. FONDEF de Conicyt. (VIU16P0011). Jefe de Proyecto.

Postulated Project:

2017 Adjudicación de Proyecto VIU (Segunda etapa). Encapsulado como inductor del crecimiento de lechuga (*Lactuca sativa* L) basado en compuestos orgánicos volátiles. Sexto concurso 2016 del programa de valorización de la investigación en la universidad del fondo de fomento al desarrollo científico y tecnológico. FONDEF de Conicyt. (VIU16P0011). Jefe de Proyecto.